

**The Role of SRGAP2 in Modulating Synaptic Dynamics in Adult Sensory Cortex**

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## **ABSTRACT**

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Human brain evolution granted us cognitive and behavioral capabilities that are unique amongst animals. SRGAP2 is a gene that was specifically duplicated in the human lineage and plays roles in the regulation of cortical development and synapse dynamics. As paralogs of one of the few known genes that regulates excitatory and inhibitory synapses concurrently, the duplications of SRGAP2 were well-positioned during human evolution to gain novel functions leading to the cognitive and behavioral phenotypes exhibited in humans. SRGAP2C, a human-specific paralog of the ancestral SRGAP2 gene, inhibits every known function of SRGAP2 and induces a phenotype similar to SRGAP2 knockdown. This induces neoteny in the maturation of synapses in mice, allowing us to study a putatively “human-like” phenotype in the mouse brain. While studies have been conducted on the effects of SRGAP2 manipulation in juvenile and young adult mice, its effects on older mice has yet to be determined.

In this dissertation, we perform longitudinal imaging experiments to determine the effects of SRGAP2 manipulation in the cortex of adult mice. In Chapter 3, we first examine the effects of SRGAP2 knockdown on the spine dynamics on apical dendrites of layer 5 pyramidal cells in the barrel cortex of adult mice, determining how it regulates spine density, turnover, and survival at baseline and in response to sensory deprivation. In Chapter 4, we study how SRGAP2 knockdown affects the clustered formations of new dendritic spines on the apical dendrites of layer 5 pyramidal cells in the barrel cortex of adult mice. Together, these results represent the first demonstration of SRGAP2 regulating on synapse dynamics *in vivo* and show that SRGAP2 knockdown can be used to model human brain evolution in adult mice.

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## **List of abbreviations**

2PSLM - 2-photon scanning light microscopy

AMPA receptor -  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor

E# - embryonic day #

E/I Ratio - excitatory/inhibitory ratio

EPSC - excitatory postsynaptic current

EPSP - excitatory postsynaptic potential

F-BAR domain - Fes-Cip4-Bin/Amphiphysin/Rvs domain

GABA - gamma-amino butyric acid

GAP - GTPase activating protein

GE - ganglionic eminence

GFP - green fluorescent-protein

IP - intermediate progenitor

LP - leading process

LTD - long-term depression

LTP - long-term potentiation

MD - monocular deprivation

NMDA receptor - *N*-methyl-*D*-aspartate receptor

P# - postnatal day #

PN - pyramidal neuron

PSD - Post-synaptic density

RG - radial glia

SH3 domain - SRC homology 3 domain

SRGAP2 - slit-robo GTPase activating protein 2

SVZ - subventricular zone

Thy1 - thymocyte differentiation antigen 1

VGCC - voltage-gated calcium channel

VZ - ventricular zone

WT - wild-type

YFP - yellow fluorescent protein

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# **CHAPTER 1**

## Introduction

Early in postnatal development, the brain is highly plastic and adaptive to environmental conditions. As animals proceed from infancy into adolescence and adulthood, the brain's capacity for structural change declines (**Lütcke et al. 2013, Koleske 2013**); however, our ability to form new memories in adulthood clearly demonstrates that plasticity is not lost in the adult brain. A central problem in neuroscience is determining the changes that occur during the maturation process, and whether these changes can be manipulated and reversed. Investigating changes in plasticity with age will enhance our understanding of age-related memory loss and neuropathology, which is critical for improving the quality of life of an aging population.

Circuitry and plasticity in neural ensembles must simultaneously accommodate two opposing needs - the ability to achieve stable representations (memory, skills), and to flexibly adapt to environmental demands. In adulthood, neuronal populations are generally stable, with no loss or gain at the cellular level (**Lütcke et al. 2013**; notable exceptions exist in the olfactory bulb and dentate gyrus, **Sahay et al. 2011, Aimone et al. 2011**). However, the properties of individual neurons are highly dynamic; frequent changes in their expressed neurotransmitters, receptors, and ion channels contribute to the modulation of synaptic strength and connectivity (**Koleske 2013**). How the properties of neural circuitry allow them to reconcile the demands of stability and flexibility is not well-understood.

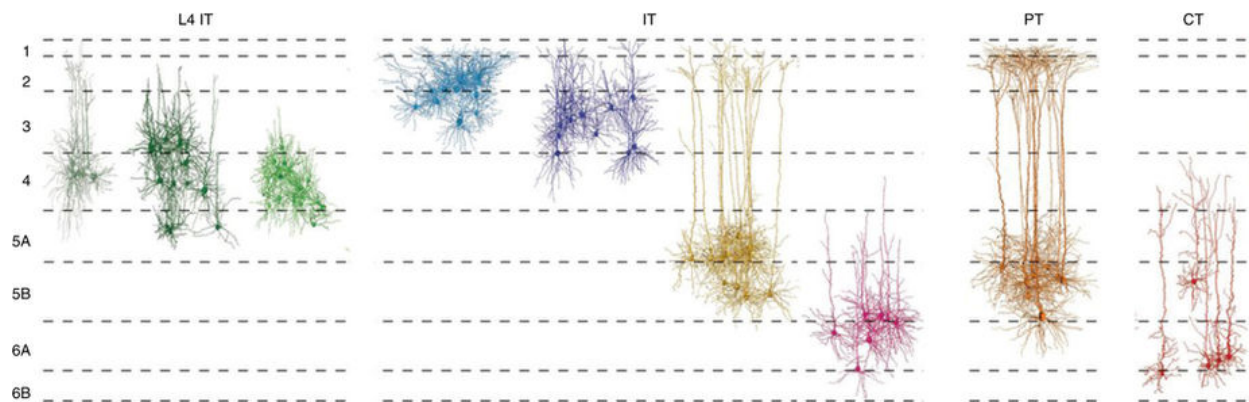
Structural plasticity in neural circuits is thought to underlie the brain's ability to form memories and adapt to environmental demands (**Holtmaat and Caroni 2016**). The features and dynamics of this plasticity varies between species, ages, brain areas, and cell types. In this dissertation, I seek to address how manipulating the gene SRGAP2 (slit-robo GTPase activating protein 2) in adult mice can modulate synaptic dynamics and plasticity in the cortex into a more "human" phenotype by delaying some features typically seen in the maturation process.

Therefore, in this chapter, I will provide an overview of the cerebral cortex, synapses, and SRGAP2 as they apply to my aims.

## 1. Cortex

The cognitive abilities that set humans apart from other species are largely attributed to the cerebral cortex. In humans, it represents ~82% of the mass of the central nervous system and 20% (~16 billion) of its neurons, with much of the remainder in the cerebellum (~80% or 69 billion neurons; **Sousa et al. 2017**)

In this chapter, I will focus on the excitatory neurons of the cortex, as inhibitory neurons are a vast subject on their own and largely outside the scope of this dissertation.



**Figure 1.1 - Heterogeneity of pyramidal neurons of the 6-layered neocortex**

Excitatory cell types in a barrel column. L4 IT shows the three morphological classes of layer 4 intratelencephalic (IT) neurons: pyramidal, star pyramidal and spiny stellate cells. Under IT are other intratelencephalic neurons of layers 2, 3, 5A/B and 6. PT shows pyramidal tract neurons of layer 5B. CT shows corticothalamic neurons of layer 6. Figure from **Harris and Shepherd 2015** (adapted from **Oberlander et al. 2012**)

### 1.1. Cortical organization

Before mammalian evolution occurred, the cortex of vertebrates often contained three layers, comparable to layers 1, 5, and 6 of mammal neocortex (**Ulinski 1974, Goffinet 1983,**

**Cheung et al. 2007, Weissman et al. 2003**). Evolutionarily, this “primitive” cortex is analogous to the allocortex present in human brains, the prime examples of which are the hippocampus and the olfactory bulb (**Stephan 1975**). In mammals, the vast majority of cortex is neocortex, which comprises six distinct anatomical layers and is composed of heterogeneous subpopulations of excitatory and inhibitory neurons (**Figure 1.1**).

The general cellular architecture of neocortex is broadly similar not only between species, but also between different cortical areas (e.g., somatosensory and motor). The neocortex is extensively interconnected in a highly specific manner; a 1 mm<sup>3</sup> volume of rodent neocortex contains approximately 10<sup>5</sup> neurons with ~4 km of axons and ~0.4 km of dendritic arborization (**Braitenberg and Schuz 1998, Oberlander et al. 2012**). Modeling suggests that these neurons could form upward of 10<sup>10</sup> connections, but found in this volume are “only” 10<sup>9</sup> synapses, perhaps more than half of which originate from outside sources (**Braitenberg and Schuz 1998**). Additionally, rather than forming a single connection (as in basic modeling), presynaptic axons often connect to postsynaptic neurons through several (e.g., 4-5) synapses (**Feldmeyer 2012**).

The most abundant general subtype of neurons, pyramidal cells, are glutamatergic neurons with pyramid-shaped somata which make up ~80% of cortical neurons (**DeFelipe and Farinas 1992, Harris and Shepherd 2015**). Each receives thousands of both excitatory and inhibitory inputs, and communicate with other cortical or subcortical regions via long-distance axonal projections in a stereotyped fashion (**DeFelipe and Farinas 1992**). Found within this subtype is incredible diversity; each sublayer of cortex has varying input and output patterns, differing gene expressions, and anatomical stereotypes (**DeFelipe and Farinas 1992, Molyneux et al. 2007**). With modern genetic techniques, still further differences among the pyramidal neurons of a single layer are being elucidated (**Zeisel et al. 2015**). Even stereotypically simple

circuits, such as the hippocampal output node, exhibit heterogeneity and complexity upon careful dissection. For example, the pyramidal cell layer of CA1 can be functionally and anatomically subdivided (**Danielson et al. 2016, Mizuseki et al. 2011, Slomianka et al. 2011**). Functional recording/imaging of pyramidal cells also are revealing the heterogeneity of pyramidal cell responses (e.g., **Pinto & Dan 2015**). Additionally, computational neuroscience is advancing toward systems to functionally classify different types of neurons (**Sharpee 2014**).

Pyramidal cells can generally be divided into 3 categories based on their axonal projections. Intracortically-projecting neurons are located in layers 2/3 (with some in layer 5) and project only within the forebrain, mostly to other cortical areas (both feedback and feedforward cortico-cortical projections) and to other structures such as the amygdala and claustrum. These are the only pyramidal cells to project to the contralateral cortex (callosal projections) in addition to their ipsilateral processes. Neurons found in layer 5-6 project mostly to subcortical structures in an area-specific manner; for example, in the primary somatosensory cortex and somato-motor areas, pyramidal neurons in layer 5B project to the spinal cord (corticospinal neurons), while in the visual cortex, pyramidal cells in layer 5B project to the superior colliculus and the pons. These neurons may also project to locations in the ipsilateral cortex. Finally, corticothalamic neurons are located in layer 6, where they project mainly to the ipsilateral thalamus (**Harris and Shepherd 2015**). Each class can be further subdivided, and have stereotyped area-specific projection patterns (**Figure 1.1**; see **Harris and Shepherd 2015** for more details, **Allene et al. 2015, Petreanu et al. 2009**). Likely related to these patterns, neurons of separate layers differentially control the network dynamics of the brain (**Beltramo et al. 2013**).

In addition to the specific hodology of pyramidal cells on the scale of brain areas and layers, inputs to these neurons are made on specific and stereotyped subcellular locations. Their

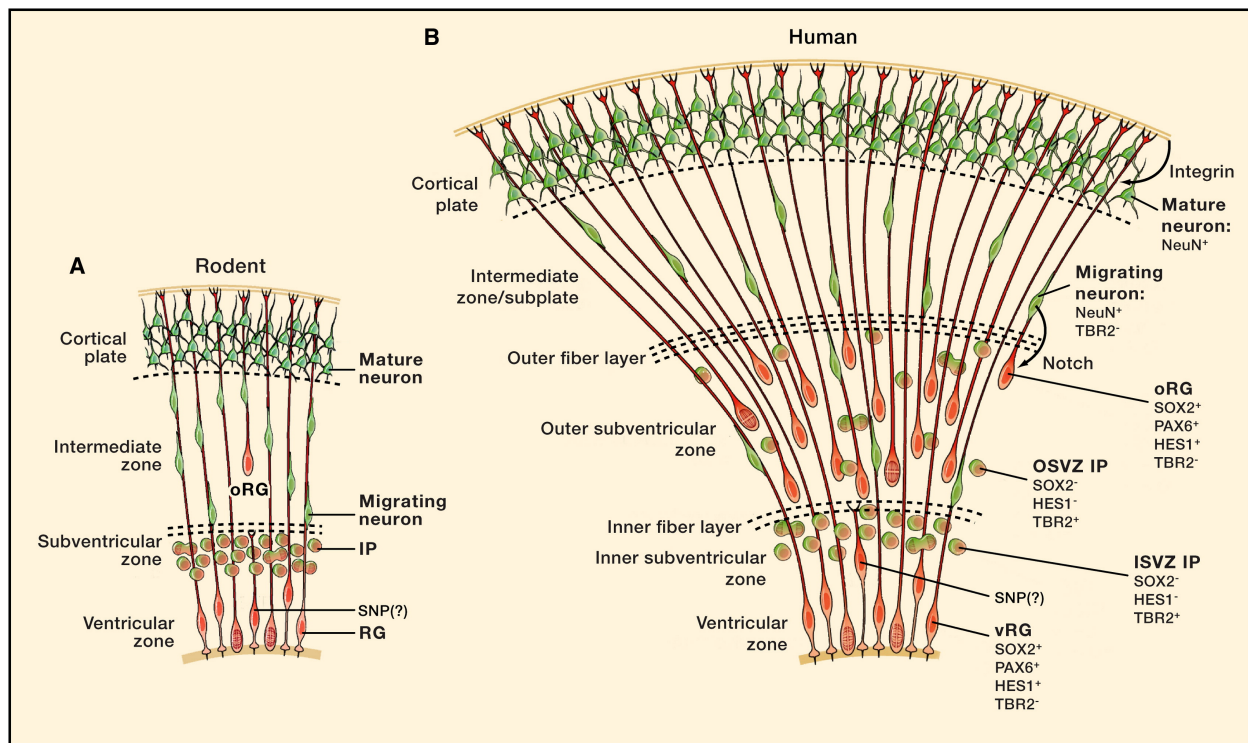
axonal outputs show similar specificity to the subcellular compartments of postsynaptic cells (**DeFelipe and Farinas 1992, Klausberger and Somogyi 2008**).

While the classification of cortical interneurons (inhibitory cells in the cortex) is an active topic of debate, they can generally be grouped into main classes by their morphology and the subcellular locations targeted by their axons. For example, large basket cells largely make perisomatic contacts and mostly express Parvalbumin, while dendritically targeting interneurons (e.g. Martinotti cells) mostly express Somatostatin. Additionally, there are chandelier cells which target the axon initial segment, and there are subtypes of interneurons that mostly target other inhibitory cells (i.e., functionally disinhibitory neurons). Similar to excitatory cells, these have stereotyped connection patterns both within/across cortical areas to specific cell types, and to specific subcellular compartments of their targets (**Harris and Shepherd 2015, Klausberger and Somogyi 2008**). However, inhibitory interneurons are morphologically and functionally much more diverse than pyramidal neurons (see e.g. **DeFelipe et al. 2013, Klausberger and Somogyi 2008, Wamsley and Fishell 2017**).

### ***1.2. Cortical development***

Neocortical pyramidal cells are generated from radial glia (RG), neural stem cells in the ventricular zone (VZ) which contact both ventricular and pial surfaces of the developing neocortex. In mice, cortical development begins at E11.5 when RGs in the VZ begin asymmetrically dividing into intermediate progenitors (IP) and self-regenerating into RGs (**Hartfuss et al. 2001, Noctor et al. 2002**). Some of these RGs establish the subventricular zone (SVZ), where they often undergo symmetric division into two daughter neurons (**Greig et al. 2013, Noctor et al. 2004, Haubensak et al. 2004**). Additionally, a third class of progenitors

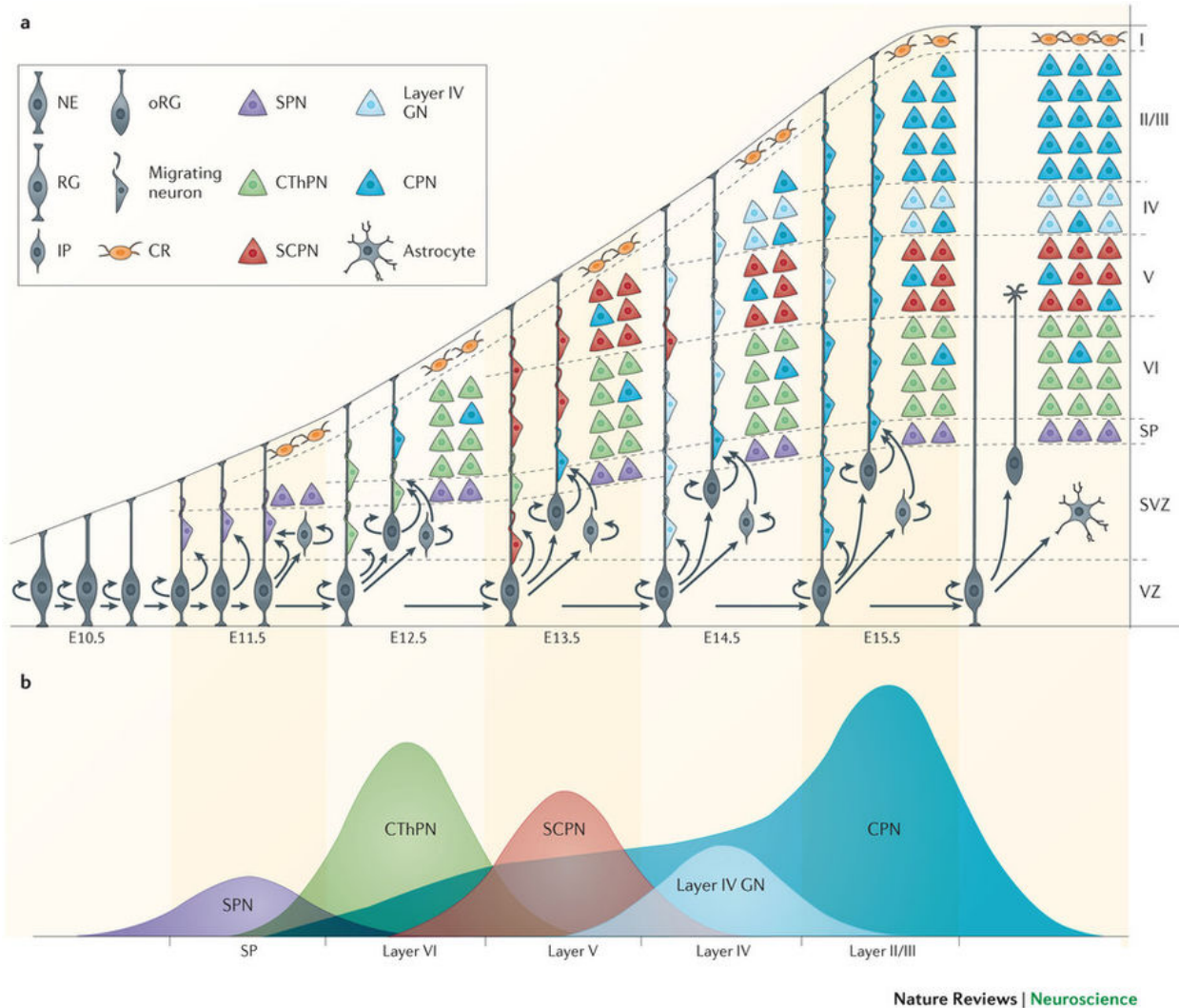
known as short neural precursors resides in the VZ and divide at the ventricular surface (Gal et al. 2006, Stancik et al. 2010; Figure 1.2A).



**Figure 1.2 - Rodent and human neocortical development**

**A.** Current views of rodent corticogenesis. Radial glia (RG) usually generate intermediate progenitor (IP) cells that divide to produce pairs of neurons, which use RG fibers to migrate to the cortical plate. **B.** In the human OSVZ (outer subventricular zone), there are oRGs (OSVZ radial glia), IP cells, and migrating neurons (red to green), with an increased number of radial fibers that neurons can use to migrate to the cortical plate. The number of ontogenetic “units” is significantly increased with the addition of oRG cells over ventricular RG (vRG) cells. Maintenance of oRG cells by Notch and Integrin signaling is shown. Short neural precursors (SNP), a transitional cell form between RG and IP cells, are also depicted in (A) and (B). Figure from Lui et al. 2011

Pyramidal neuron subtypes (see above) are born in waves over the course of neurogenesis from E11.5 through E17.5 in mice. These neurons migrate radially in an “inside-out” pattern, eventually forming the 6 layers of the neocortex (Greig et al. 2013; Figure 1.3). For more in depth discussions on cortical development, see Lui et al. 2011 and Greig et al. 2013.



**Figure 1.3 - Development of the six layers of neocortex in mice**

**A.** Radial glia (RG) in the ventricular zone (VZ) begin to produce projection neurons around E11.5. At the same time, RG generate intermediate progenitors (IPs) and outer RG (oRG), which establish the subventricular zone (SVZ) and act as transit-amplifying cells to increase neuronal production. After neurogenesis is complete, neural progenitors transition to a gliogenic mode, generating astrocytes and oligodendrocytes (not shown). Cajal–Retzius (CR) cells primarily migrate into neocortical layer I from non-cortical locations, whereas other projection neurons are born in the neocortical VZ and/or SVZ and migrate along radial glial processes to reach their final laminar destinations. **B.** Distinct projection neuron subtypes are born in sequential waves over the course of neurogenesis. The peak birth of subplate neurons (SPN) occurs around E11.5, with the peak birth of corticothalamic projection neurons (CThPN) and subcerebral projection neurons (SCPN) occurring at E12.5 and E13.5, respectively. Layer IV granular neurons (GN) are born around E14.5. Some callosal projection neurons (CPN) are born starting at E12.5, and those CPN born concurrently with CThPN and SCPN also migrate to deep layers. Most CPN are born between E14.5 and E16.5, and these late-born CPN migrate to superficial cortical layers. Peak



sizes are proportional to the approximate number of neurons of each subtype born on each day. NE, neuroepithelial cell. Figure from **Greig et al. 2013**

### ***1.3. Cortical plasticity and age***

In development, “critical periods” are periods of time where there is greatly enhanced potential for plasticity caused by sensory experiences or sensorimotor interactions. These epochs typically occur soon after birth and decline thereafter at variable rates, depending on the species and system studied (**Hübener and Bonhoeffer 2014**). The first described example of this is that of filial imprinting in birds, where fledglings are imprinted on their mothers in a few hour period soon after hatching, causing them to follow them around closely (**Lorenz 1935**). In mammals, a typical example is found in the visual system, where a classic study showed that temporary closure of one eye (monocular deprivation, MD) in cats leads to rewiring in visual cortex only during a brief period early in life (**Hubel and Wiesel 1970**).

An example of a critical period in humans is that of language acquisition potential in adolescence, which follows a stereotypical decline from infancy to young adulthood (**Kuhl 2010**). In general, critical periods in humans are thought to end around 10 years of age (**Hübener and Bonhoeffer 2014**).

While plasticity is more profound in the developing brain than later in life, it is not a feature exclusive to the young. After the critical period, synaptic plasticity remains widespread in the adult brain; several brain structures, such as the hippocampus, striatum, and cerebellum, depend on their capacity for plasticity to serve their functions (**Hübener and Bonhoeffer 2014**). However, the neocortex has canonically been considered to be plastic only during the critical period; it is only somewhat recently that studies have shown the plastic potential of neocortex later in development. For example, reorganization of the somatosensory cortex is seen following

finger amputation (**Merzenich et al. 1984**), and lesions of the cochlea causes rearrangements of the topography of the auditory cortex (**Robertson and Irvine, 1989**). Additionally, the visual cortex displays plasticity following retinal lesions (e.g. **Gilbert et al. 1990, Kaas et al. 1990, Keck et al. 2008**).

Despite these examples, inexorable cognitive decline does come with age. In humans, it was previously thought that a decrease in cortical density and deterioration of dendritic branching were the main culprits behind age-related cognitive decline. However, more recent studies have largely debunked these views and turned attention to more subtle factors, such as region-specific changes in dendritic morphology, altered connectivity, breakdowns in calcium regulation, gene expression changes, or other factors that can affect plasticity or alter the network dynamics of cognition-related circuitry (**Burke and Barnes 2006**). Below, I will specifically discuss how structural plasticity changes with age (see later section on synapses).

#### ***1.4. Cortical evolution***

The genomes of humans and chimpanzees are separated by 6-7 million years of evolution, during which we accumulated ~35 million nucleotide substitutions (1.2% of the whole genome) along with 90 million base pairs worth of structural variations, including insertions, deletions, inversions, and duplications (**Sousa et al. 2017, Tyler-Smith and Xue 2012**). Human evolution granted us the largest brain by volume and number of cortical neurons among primates; however, while the overall size of the central nervous system is correlated with general intelligence and other indicators of cognitive capacity, this does not present a complete story. For example, modern human brains can vary by 2-fold between individuals, but without significant differences in cognitive measures (**Geschwind and Rakic 2013**).

Apart from absolute size, number of neurons is also a weak measure of cognitive abilities. Other species, such as dolphins, have significantly more neurons than humans, while humans with congenital or acquired conditions where brain regions are severely underdeveloped or missing can have normal levels of intelligence and cognitive skills (**Mortensen et al. 2014, Sousa et al. 2017**). Therefore, the evolutionary bestowal of “human” qualities is not fully dependent on size or number of neurons, but rather more nuanced qualities such as molecular/biochemical changes, cell-type diversity, or expanded/more complex connectivity patterns. Some studies suggest that the expansion of the human brain led to increased cognitive abilities as it loosened constraints on the association cortices previously imposed by developmental molecular gradients and neuronal activity patterns, allowing the formation of new cortico-cortical connections and the rewiring of ancestral circuits (**Buckner and Krienen 2013, Sousa et al. 2017**). For example, the developing frontal and temporal lobes of humans form “islands” of specific gene-expression patterns distinct from the developing brains of mice and macaque, which eventually form Broca and Wernicke’s areas, regions devoted to the emergence of language (**Geschwind and Rakic 2013, Kuhl 2010**).

While the human brain exhibits more elaborate gyrification compared to its closest living relatives, such as chimpanzees and bonobos (likely as a necessary consequence of the enlarged cortical surface area), whether humans have novel brain structures, cell types, or circuits that are not present in other primates is an active area of debate (**Geschwind and Rakic 2013, Hofman 2012, Rogers et al. 2010**). With few exceptions, there are no obvious changes in the histological structure in human cortex (**Preuss and Coleman 2002**); however, several studies report human-specific changes in neuronal organization and connectivity, as well as the morphological/molecular/biochemical properties and quantity of both excitatory and inhibitory neurons and glia

(**Bianchi et al. 2013, Elston et al. 2011, Herculano-Houzel 2016, Kwan et al. 2012, Oberheim et al. 2012, Sherwood et al. 2004, Sousa et al. 2017**). Of particular importance for this dissertation, human pyramidal neurons are larger, have more complex dendritic arborization, and exhibit higher dendritic spine density compared to those of our primate cousins (**Elston et al. 2011, Sherwood et al. 2003**; see later section on dendritic spines).

Humans may also differ in their long-distance projections (**Anderson et al. 1999**). Short range intracortical and inter-areal connections are also increased, with an accompanying increase in the thickness and neuron number of layer 2/3 (where these connections originate) compared to other primates (**Hutsler et al. 2005, Marin-Padilla 1978, Rockel et al. 1980**). This is thought to cause the increased gyrification in humans compared to their primate cousins (**Van Essen 1997, Hofman 2012**).

Another known characteristic of human cortex is a high number and variety of local circuit neurons, most of which are inhibitory interneurons; some of these may be unique to humans, as they differ developmentally from interneurons in mice, as detailed below (**DeFelipe et al. 2002, Geschwind and Rakic 2013**).

Developmentally, humans and other primates have expanded proliferative zones and a larger variety of neural stem and progenitor cell subtypes, which collectively imbues the developing primate brain with enhanced proliferative capacities compared to rodents (**Bae et al. 2015, Dehay et al. 2015, Florio et al. 2015, Geschwind and Rakic 2013, Lui et al. 2011, Taverna et al. 2014**). For example, the developing primate brain contains RG-like cells found outside of the VZ which do not span the length from the ventricle to the pia (**Lui et al. 2011**). Additionally, corticogenesis in primates exhibits the appearance of a large SVZ which can be anatomically divided into inner and outer region (**Smart et al. 2002, Zecevic et al. 2005, Fish et**

**al. 2008**). A population of RG-like cells which are distinct from RG in the VZ resides in the outer SVZ, where they also function as neural progenitors; this population self-renews through asymmetric division, generating another set of IP cells within the outer SVZ. This dramatically changes the architecture of the migration scaffold formed by RGs, as their fibers no longer span the apical and basal surfaces of the cortex (**Figure 1.2B**). These factors may cause increased neuron production leading to the production of a larger brain.

In rodents, GABAergic interneurons are generated in the medial and lateral ganglionic eminences (GE) and migrate laterally to reach their final spots in the cortex (**Anderson et al. 2001**). In humans, some interneurons are generated from other regions, including the VZ/SVZ (**Anderson et al. 2001, de Carlos et al. 1996, Jakovcevski et al. 2011, Letinic et al. 2002, Petanjek et al. 2009**)

At the neuronal level, the cell cycle in primates is five times longer than in mice. However, this longer cell cycle is counteracted by a significantly extended period of cortical neurogenesis - from 6 days in mice to 60 days in the macaque monkey and ~100 days in humans (**Geschwind and Rakic 2013**). This results in a larger neocortex in humans, particularly in the upper-layers, as the timing and duration of neurogenesis correlates with the size of neocortex (**Finlay and Darlington 1995, Geschwind and Rakic 2013, Hutsler et al. 2005, Marin-Padilla 1978, Rockel et al. 1980**).

The general time scale of brain maturation is also quite different in humans compared to other mammals. Human brains retain a large degree of plasticity for two decades and beyond, which surpasses the entire lifespans of some other primates (**Silbereis et al. 2016**). The process of dendritic and synaptic maturation/elimination, along with cortical myelination, are extended until the third decade of life in humans, compared with puberty for other primates (**Bianchi et al.**

**2013, Geschwind and Rakic 2013, Huttenlocher et al. 1982).** This neoteny could result in an extended childhood/adolescence period and effectively lengthen the critical period, enabling a host of environmental factors to affect the development of our cognitive, emotional, and social capabilities (**Bogin 1994, Silbereis et al. 2016, Sousa et al. 2015**). This extended period of maturation is further emphasized by the fact that the human neonatal brain is a much smaller fraction of the mass of the eventual adult brain compared to other primates (**Bianchi et al. 2013, Robson and Wood 2008**).

Development of the human brain also displays heterochrony - different developmental timelines for different cortical regions. For example, synaptic elimination is not synchronous across regions in humans compared to chimpanzees (**Geschwind and Rakic 2013**); additionally, in both humans and chimpanzees, the maturation of dendritic arborization is delayed in the frontal cortex compared to both sensory and motor regions (**Bianchi et al. 2013**). Similarly, some association areas like the prefrontal cortex exhibit neoteny and prolonged development, which may be linked to various neuropsychiatric disorders due to inappropriate synaptic pruning and increased vulnerability to defects due to the prolonged development (**Paus et al. 2008, Selemon et al. 2013**).

## **2. Synapses**

The human central nervous system has approximately 86 billion neurons, with between several hundred trillion and well over a quadrillion synapses, which can be defined as the presence of a presynaptic active zone with synaptic vesicles, a well-defined synaptic cleft, and a postsynaptic density (**Holtmaat and Svoboda 2009, Sousa et al. 2017**). The connections utilize a variety of

neurotransmitters to communicate varying information, and the connectivity patterns are an active area of research.

### ***2.1. Excitatory synapses***

Excitatory synapses in the central nervous system function to depolarize the postsynaptic cell and possibly trigger action potentials. In the central nervous system, glutamate is the canonical neurotransmitter released by presynaptic cells and binds both ionotropic AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate), NMDA (*N*-methyl-D-aspartate), and Kainate receptors and metabotropic glutamate receptors on the postsynaptic cell.

Excitatory inputs onto pyramidal cells are marked by dendritic spines, which are membrane protrusions on their dendrites that are the site of connections with presynaptic cells. Further details on excitatory synapses will follow (see section below on dendritic spines).

### ***2.2. Inhibitory synapses***

Inhibitory synapses function to prevent action potential formation in the postsynaptic cell, often through hyperpolarizing or shunting inhibition. They make up 10-15% of total synapses in the neocortex, and are difficult to isolate biochemically and morphologically when compared with their excitatory counterparts (which are generally marked by dendritic spines; see above and below; **Fossati et al. 2016**). As such, there is a relative dearth of studies on inhibitory synapses compared with excitatory synapses, with much more known about the molecular mechanisms behind excitatory synapse development (**McAllister 2007, Shen and Scheiffele 2010**).

The physical location of inhibitory synapses on neurons is of great importance; different types of inhibitory neurons will synapse onto different compartments of pyramidal neurons (**Hu**

**& Vervaeke 2017, Kubota et al. 2016**). The classical concept of inhibition onto cortical pyramidal cells is that of axo-somatic inhibition, where inhibitory neurons synapse directly onto the soma. However, these make up a small fraction of the total synapses formed by inhibitory interneurons (~10%; **Kubota et al, 2016**). Inhibitory inputs onto dendrites comprise much more of the total inhibitory output in the cortex, and can also vary from perisomatic (where they can prevent the majority of excitatory signals from reaching the soma) and much more distal (where they might inhibit specific synaptic inputs; **Kubota et al. 2016**). Many inhibitory interneurons also synapse onto dendritic spines (see section below on dendritic spines), where they can both affect the electrophysiological properties of the spine and have important effects on their development and dynamics (**Chen and Nedivi 2013, Chiu et al. 2013, Kubota et al. 2016, Müllner et al. 2015, Oh et al. 2016, Villa et al. 2016**).

In the forebrain, most inhibitory synapses contain type A GABA receptors postsynaptically, which are stabilized by a lattice of Gephyrin molecules under the plasma membrane (**Tretter et al. 2012**). Gephyrin is a core component of the inhibitory postsynaptic scaffolding, and forms high-order polymers which have binding sites for most identified components of inhibitory synapses, including type A GABA receptors (**Fritschy et al. 2008, Tretter et al. 2012, Tyagarajan and Fritschy 2014**).

### ***2.3. Excitatory-inhibitory balance***

The balance between excitatory and inhibitory activity in the brain is critical for proper neural systems function. The ratio of excitatory to inhibitory activity (E/I ratio) is conserved between rodents and humans, despite the much higher density of cortical synapses in humans (**DeFelipe 2011**). Additionally, the E/I ratio is achieved early in development, well before synaptic density

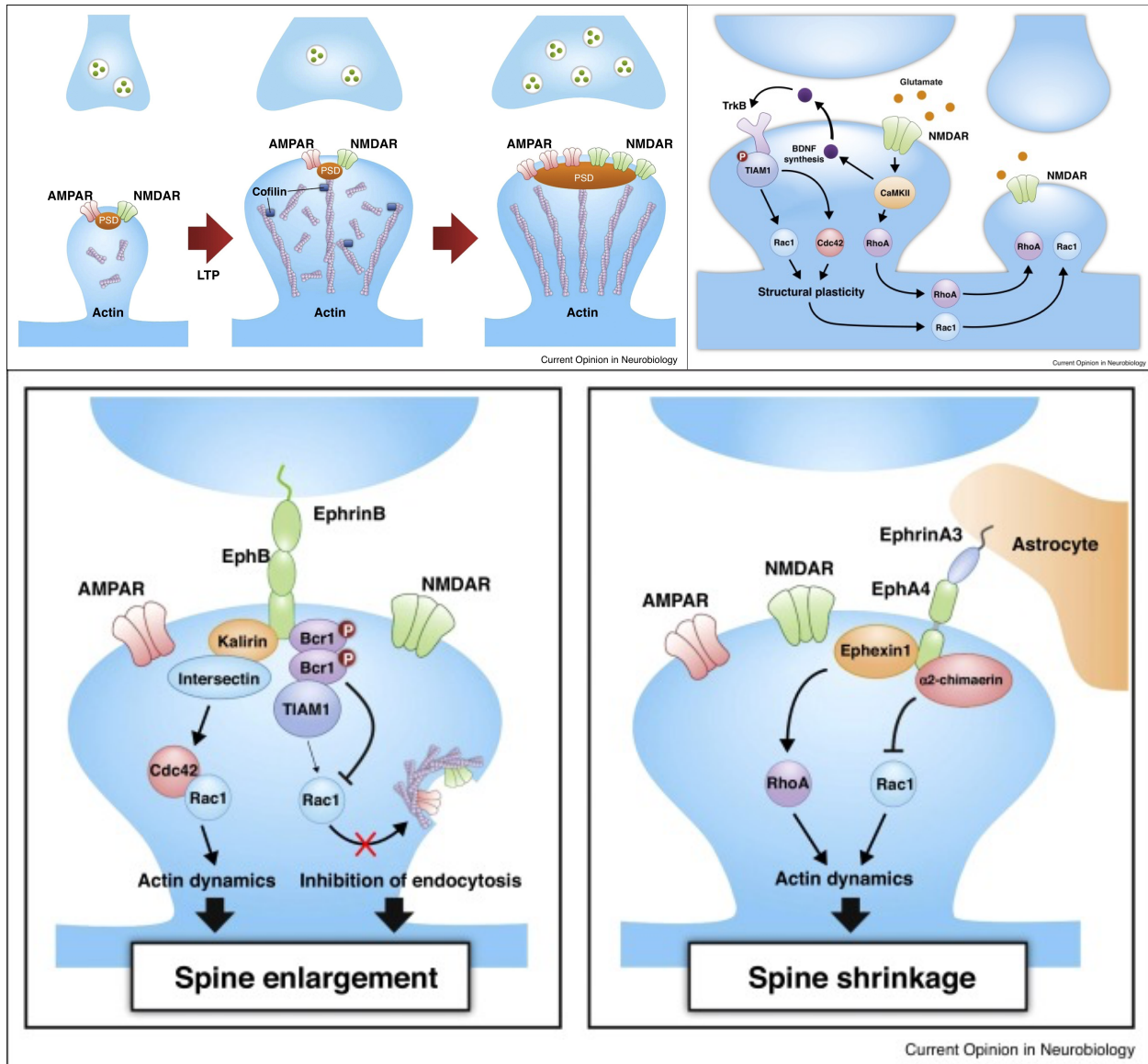


reaches mature levels (**Benson and Cohen 1996, Soto et al. 2011, Zhao et al. 2005**). Indeed, the E/I ratio of individual cells can reach final levels before the onset of sensory experience, suggesting that the ratio is set by developmental mechanisms without the need of any environmental influence (**Soto et al. 2011**). Interestingly, there are few genes known to be involved in the assembly of both excitatory and inhibitory synapses, despite the tight regulation clearly necessary to achieve the E/I ratio (**Fossati et al. 2016**; see section below on SRGAP2).

#### ***2.4. Dendritic spines***

In 1888, Ramon y Cajal described the presence of small protrusions, resembling “bristling thorns or short spines,” on the surface of Golgi-stained cerebellar Purkinje cells in birds (**Chen et al. 2014, DeFelipe 2015, Yuste 2015**). These membrane protrusions are now known as dendritic spines, and are the termination site of over 90% of excitatory synapses in the brain, giving the human brain over  $10^{13}$  spines (**Nimchinsky et al. 2002**). Dendritic spines consist of a  $\sim 1 \mu\text{m}$  head with volumes ranging from  $0.001\text{-}1 \mu\text{m}^3$  and a thin  $\sim 0.1 \mu\text{m}$  neck with lengths that can vary from  $0.1$  to  $2 \mu\text{m}$  (**Harris and Kater 1994, Nimchinsky et al. 2002**).

Dendritic spines are often used as proxies for excitatory synapses postsynaptically. This practice is supported by the fact that spines persisting for  $>15\text{-}19$  hours consistently show the ultrastructural hallmarks of typical synapses when examined under electron microscopy (**Knott et al. 2006, Nagerl et al. 2007**). Additionally, in an electrophysiological study of rat hippocampal slice cultures, both AMPA- and NMDA receptor currents of hours-old spines are indistinguishable from currents measured from mature spines of comparable volume (**Zito et al. 2009**). However, it is important to note that in certain cases, dendritic spines may not always possess functioning excitatory synapses (**Sando et al. 2017**).



**Figure 1.4 - Molecular composition of dendritic spines**

**A.** Structural synaptic plasticity is initiated by the coordinated growth of dendritic spines and increased actin within the dendritic spines. The formation of stabilized structural and functional synapses requires initial spine growth, followed by an increase of postsynaptic density and subsequent presynaptic boutons. **B.** Local BDNF–TrkB–Rho GTPase signaling is required for synaptic crosstalk. Synaptic activity stimulates the local synthesis and release of brain-derived neurotrophic factor (BDNF), which induces the activation of its receptor, TrkB, at the same spines and results in the activation of the Rho GTPases, Cdc42 and Rac1. Only activated Rac1, together with activated RhoA stimulated by NMDA receptor, will be transported to the neighboring dendritic spines. The spread of active Rac1 and RhoA into the neighboring spines primes the spines to undergo structural plasticity even when a weak stimulus is received. **C.** Different signaling cascades can modulate the enlargement or shrinkage of dendritic spines. Ephrin-dependent EphB stimulates different guanine nucleotide exchange factors (GEFs) at the dendritic spines during spine enlargement. The dynamic control of Rho GTPase signaling is

critical for the structural plasticity. EphA4 is suggested to enhance RhoA activation through the concerted regulation of GEF and GTPase-activating protein (GAP) activity. Figure from **Fu and Ip 2017**

#### *2.4.1. Molecular composition of dendritic spines*

As the postsynaptic sites of excitatory synapses, dendritic spines are locations where AMPA- and NMDA-receptors are concentrated to allow efficient excitatory transmission (**Figure 1.4A, Grienberger et al. 2015**). Spines possess an intricate molecular machinery to perform their functions and regulate their morphology (**Bourne and Harris 2008, Fu and Ip 2017, Sheng and Hoogenraad 2007**). There is local translation of mRNAs coding for ion channels, neurotransmitter receptors, adhesion molecules, scaffolding proteins, signaling molecules, cytoskeletal proteins, and translation/degradation machinery (**tom Dieck et al. 2014**).

The main cytoskeletal component of dendritic spines is filamentous (F-) actin (**Okamoto et al. 2004, Konietzny et al. 2017**). Spine morphology is tightly regulated by proteins controlling F-actin dynamics; actin polymerization and stable pools of F-actin increases rapidly during spine enlargement and promotes the anchoring of synaptic proteins within the spine.

Actin polymerization is regulated by both extra- and intracellular signals that converge onto small GTPases of the Rho family, which in turn modulate the activity of specific actin-binding proteins which can polymerize or sever F-actin (**Govek et al. 2005**). In particular, the Rho GTPases RhoA, Rac1, and Cdc42 are known to be heavily involved in spine morphogenesis (**Figure 1.4B, Lai and Ip 2013, Sala and Segal 2014, Woolfrey and Srivastava 2016**). On a simplistic level, Rac1 and Cdc42 activation stimulates F-actin polymerization and promotes spine formation and enlargement, while RhoA activation causes spine shrinkage through its effector RhoA kinase (**Figure 1.4C, Saneyoshi et al. 2010**). However, the effects of each

individual GTPase depends on the relative levels of expression of activity of each within spines (**Sala and Segal 2014**). Additionally, the Ras family of small GTPases and the related Rap GTPases are also involved in regulating spine morphogenesis (**Sala and Segal 2014**).

When LTP is induced in spines, activation of NMDA receptors leads to local calcium influx which activates CamKII, causing a cascade effects leading to the activation of Rho GTPases and changing the composition of actin-binding proteins in the spine (**Meng et al. 2002, Tolia et al. 2005, Tolia et al. 2011, Xie et al. 2007**). Cofilin, which regulates actin dynamics, accumulates rapidly within the spine (**Bosch et al. 2014**). The effect of cofilin on actin polymerization is concentration-dependent; at low concentrations, it can sever actin filaments and generate new barbed ends for additional actin growth, and at higher concentrations it can enhance F-actin nucleation and polymerization (**Adrianantoandro and Pollard 2006, Spence and Soderling 2015**). The increase of F-actin in LTP-affected spines leads to spine enlargement and increases the number of docking sites in the spine, which can bind the proteins newly translated by the induction of LTP (**Okamoto et al. 2009**). This includes the recruitment of more AMPA receptors, increasing the strength of the synapses (**Lamprecht and LeDoux 2004**).

Receptor tyrosine kinase activity can also activate Rho GTPases to modulate actin activity. In hippocampus, brain-derived neurotrophic factor (BDNF) can bind tropomyosin receptor kinase B (TrkB) to activate Rac1 and modulate cofilin activity (**Figure 1.4B, Valnegri et al. 2015**). Another receptor tyrosine kinase family, Eph (erythropoietin-producing hepatocellular) receptors, also has roles in dendritic spine morphology through their activation of Rac1 and Cdc42 through recruitment of upstream effectors (**Fu and Ip 2017**).

Additionally, various G-protein coupled receptors (GPCRs) have been linked to spine morphogenesis. For example, the melanocortin 4 receptor (MC4R) can be activated by

endogenous ligands to activate actin regulators like Tiam1, WAVE1, and RhoA, along with causing local AMPA receptor insertion (**Shen et al. 2013**).

Apart from actin, many structural proteins are located in the postsynaptic density (PSD), the assembly of scaffold proteins that link synaptic receptors and membrane proteins to the actin cytoskeleton and various signaling molecules (**Sala and Segal 2014**). The most abundant scaffolding protein is PSD-95, which plays important role in synapse/spine formation through interactions with proteins that mediate transsynaptic adhesion (**Sala and Segal 2014**). PSD-95 also interacts with numerous signaling molecules that allow membrane receptors to activate cytoplasmic effectors (**Sala and Segal 2014**).

Two other classes of scaffolding proteins, Homer and Shank, are also major regulators of spine morphology (**Hayashi et al. 2009, Meyer et al. 2014, Sala and Segal 2014**). These form a mesh-like matrix structure which serve to anchor other synaptic proteins, and can interact with various regulators of spine morphology such as Cdc42 (**Hayashi et al. 2009**).

Cell-adhesion molecules are also important in spine dynamics. For example, the synaptic cell-adhesion molecule SynCAM1 stabilizes nascent synaptic contacts and promotes their maturation, and its loss of function leads to a decrease in spine survival rate (**Körber and Stein 2016**). Other cell adhesion molecules involved in dendritic spine formation include Neuroligin, SALM, netrin-G ligand, and IL1RAPL1, all of which bind to PSD-95 (**Sala and Segal 2014**). Additionally, extra-cellular matrix proteins are also important in regulating spine dynamics (see Chapter 5).

Finally, apart from ionotropic glutamate receptors for binding neurotransmitters, spine heads can contain mGluR and nicotinic cholinergic receptors (**Sala and Segal 2014**). These, along with the previous receptors mentioned, can activate a variety of effector molecules like

PKA and ERK (**Tang et al. 2017**). Additionally, spines can receive multiple synaptic inputs simultaneously (**Chen et al. 2012, Chiu et al. 2013, Villa et al. 2016**).

There are several more molecular components of spines that are outside the scope of this dissertation. For more extensive reviews on the molecular makeup of dendritic spines, see **Dotti et al. 2014, Sala and Segal 2014** and **Konietzny et al. 2017**.

#### *2.4.2. Classification of dendritic spines*

Classically, many anatomists have attempted to categorize protrusions from dendritic shafts (i.e., dendritic spines) into morphological categories - e.g., filopodial, thin, cup-shaped, stubby, or mushroom spines (**Fu and Ip 2017**).

However, the advent of new imaging techniques such as super-resolution stimulated emission depletion (STED) imaging have revealed that spine morphology exists on a continuous gradient, rather than forming discrete categories (**Tønnesen et al. 2014**). This confirms what several researchers have long-argued, lending credence to their decision to treat all dendritic protrusions as spines (**Fu and Zuo 2011**). However, there remains debate in the field about discrete subtypes of dendritic protrusions, and in particular the distinction between filopodia and dendritic spines. In many studies, spines are identified as protrusions with bulbous heads with a neck, while filopodia may be described as long, thin protrusions without clearly defined heads, which are much more dynamic than spines (e.g., **Lendvai et al. 2000, Grutzendler et al. 2002, Xu et al. 2009, Yang et al. 2009, Zuo et al. 2005**).

The size of dendritic spine heads correlates well with the size of the postsynaptic density, the number of synaptic AMPA glutamate receptors, and the number of presynaptic vesicles; as

such, it is a good indicator of synapse maturation and strength (**Arellano et al. 2007, Bourne and Harris 2007, Harris and Stevens 1989, Matsuzaki et al. 2004**).

#### *2.4.3. Biophysical properties of dendritic spines*

The physical properties of dendritic spines are inextricably tied to their functions. In a landmark early study, Yuste and Denk found that spine membranes contain voltage-gated calcium channels (VGCCs) which can be activated both through synaptic input and through backpropagating action potentials from the soma, which can invading the spine with little loss in amplitude (**Kwon et al. 2017, Yuste and Denk 1995**). Importantly, when synaptic stimulation was paired with somatic spikes, the calcium signal from both summed supralinearly, providing evidence that spines can serve to detect temporal coincidence of pre- and postsynaptic activity, thus serving as basic functional units of neuronal integration (**Yuste and Denk 1995**).

The dimensions of the spine neck and head both serve the properties of a given spine. The small volume of the spine head is proposed to mediate efficient information transfer by permitting the efficient increase of calcium concentrations with a low intensity of input by the presynaptic cell (**Fujii et al. 2017**). Additionally, the volume of the spine head is directly linked with the size of the PSD, the number of vesicles in the presynaptic axon bouton, and synaptic strength, while the spine neck acts as a physical barrier between the synaptic machinery in the spine head and the dendritic shaft, whose length impacts the effective compartmentalization of synaptic signaling and input filtering (**Bourne and Harris 2008, Harris 1998, Higley and Sabatini 2008, Yuste 2011**). Along with spine neck length, the spine density along a dendrite also contributes to the compartmentalization between synapses and dendrites in cortical pyramidal neurons (**Higley and Sabatini 2008, Yuste 2013**).

Dendritic spines possess a host of voltage-gated channels beyond VGCCs, including those for sodium and potassium, giving them excitable membranes. Spines can be effective at compartmentalizing voltage changes within the synapse by behaving as electrical compartments, reducing the synaptic input to the cell and attenuating EPSPs (**Kwon et al. 2017**). Shorter spine necks attenuate less current, producing larger somatic EPSCs in response to excitatory input (**Araya et al. 2014**). Longer spines, on the other hand, may not allow synaptic input to reach the soma at all; their function may be integrate into circuits later by transforming into shorter spines when exposed to plasticity-producing stimuli (**Sala and Segal 2014**). Estimates of spine neck resistance range from 1.2 G $\Omega$  to 4-50 M $\Omega$ , which is critical for the ability of spine heads to generate large currents; in some cases, spine heads may be able to generate large voltage transients that are sufficient to sensitize nearby spines, priming them for LTP (**Sala and Segal 2014**). Additionally, spines can effectively compartmentalize the GABAergic inhibition generated by inhibitory synapses that target spine heads (**Chiu et al. 2013**).

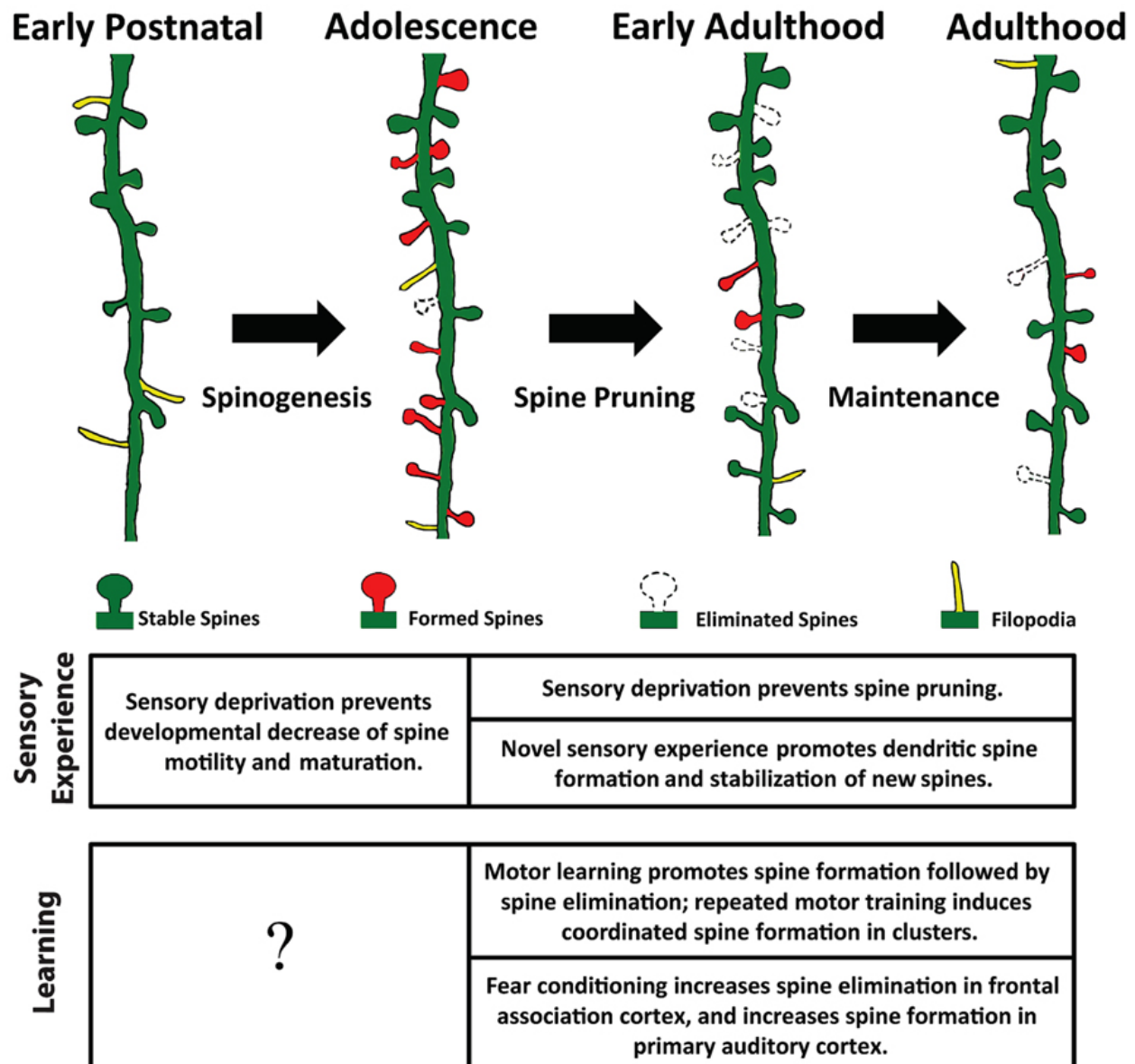
The spine neck is also effective at restricting calcium changes within the spine, limiting leakage into the neighboring dendritic shaft (**Alvarez and Sabatini 2007, Noguchi et al. 2005**). This can be important both for limiting calcium signaling outside a small area (the spine) and for generating larger concentrations of calcium. The magnitude of compartmentalization is modulated by spine neck size, as one study showed a 15 fold difference in the peak spine head concentration of calcium between spines with 0.1  $\mu\text{m}$  and 0.55  $\mu\text{m}$  diameter necks (**Gold and Bear 1994**). As such, the degree of NMDA receptor-driven LTP driven by calcium influx has direct relationships with spine-neck geometry (**Noguchi et al. 2005**).

Apart from voltage and calcium, spines can also be effective at confining second messengers and enzymes (**Svoboda et al. 1996**). However, this depends on the molecule in



question; while Cdc42 activity remains largely within the spine head (**Murakoshi et al. 2011**), Ras and Rho GTPases are known to escape the confines of the spine (**Harvey et al. 2008**, **Murakoshi et al. 2011**). This can also depend on spine neck size, as larger-necked spines can let messengers into the dendritic shaft more efficiently (**Ebrahimi and Okabe 2014**). This suggests that spines with different neck sizes may serve different functions in the dendritic arbor. However, the diffusion of molecules across the spine neck depends on more than simply spine neck size, as different molecules may be bound by anchoring molecules within the spine, preventing their escape (**Ebrahimi and Okabe 2014**). Additionally, diffusion across the spine neck is regulated bidirectionally by both pre- and postsynaptic activity, with active neurons able to restrict diffusional coupling between spines and their parent dendrites (**Bloodgood and Sabatini 2005**). This could serve as a mechanism to concentrate plasticity-inducing molecules within the spine head of active synapses in another example of spines as functional units of integration.

Finally, theoretical and computational neuroscience has, in the last few decades, begun to integrate experimental findings of dendrites and dendritic spines into models. Early theoretical views of dendritic spines and dendrites treated them as passive cylindrical compartments. Eventually, the electrotonic distance of synapses to the soma was considered, and the importance of dendritic spines having excitable membranes became recognized (**Sala and Segal 2014**). The physical properties of dendrites and dendritic spines are important factors in divining the logic behind how neural networks effect plasticity and perform computations, a topic which is beyond the scope of this dissertation (see **Mel et al. 2017 for some discussion on this subject**).



**Figure 1.5 - Dynamics of dendritic spines**

Spine remodeling at different stages of an animal's life. Rapid spinogenesis in early postnatal is followed by a gradual spine pruning in adolescence. In adulthood, spine formation and elimination reach equilibrium, with a small fraction of spines constantly added or removed. Experience affects spine dynamics differently at different developmental stages. Figure from Chen et al. 2014

#### *2.4.4. Developmental progression of dendritic spines*

In early development, a large proportion of dendritic protrusions are filopodia, which are long, thin, and motile membrane protrusions (**Ozcan 2017**). This population decreases throughout maturation; in mouse barrel cortex, the proportion decreases from >50% at ~P15 to <10% at ~P30 (**Zuo et al. 2005**). At juvenile ages (~P15), dendritic spines and filopodia are very dynamic, exhibiting various structural changes (appearance, disappearance, or morphological evolution) on the time scale of minutes (**Lendvai et al. 2000**). In contrast, by ~P30, spines and filopodia have reported diverged dynamically, with filopodia displaying turnover daily while spines remain more stable (**Grutzendler et al. 2002, Zuo et al. 2005**). By the age of 4 months, >70% spines are reported to be stable over 18 months (**Figure 1.5, Grutzendler et al. 2002, Zuo et al. 2005**)

Additionally, an enormous surplus of spines are formed early in development. Between 1 and 4 months of age, cortical spines in general go through a stage of immense pruning, with ~30% of all spines being lost (**Grutzendler et al. 2002, Zuo et al. 2005**). After this stage, spine elimination becomes comparable to the rate of spine formation, though there remains some overall spine loss throughout adulthood (**Grutzendler et al. 2002, Zuo et al. 2005**).

In rodent barrel cortex, layer 2/3 neurons have spines that gradually decrease in motility (elongation or shortening of spines) and turnover rate in adolescence, while spine density concomitantly increases (**Lendvai et al. 2000, Cruz-Martinet al. 2010**).

#### *2.4.5. Cortical spines*

Within a given cortical pyramidal neuron, spines can have varying properties (**Nimchinsky et al. 2002**). Spine density, for example, can vary from 0.2 to 3.5 spines per  $\mu\text{m}$  of dendrite (**Sala and Segal 2014**). In the rabbit cerebral cortex, the apical dendritic shafts have twice the spine density

of basal dendrites (**Globus and Scheibel 1966, Globus and Scheibel 1967**). For most reported cases in rats, monkeys, and humans, spine density is higher in apical dendritic shafts than in apical tufts/oblique branches and basal dendrites (**Feldman and Dowd 1975, Kemper et al. 1973, Marin-Padilla 1967, Parnavelas et al. 1973**). In contrast, at least one study reported no significant differences in spine density in mouse dendritic segments, though this is contradicted by others (**Ballesteros-Yáñez et al. 2006**, see below). Importantly, spine distribution may not be uniform along a given dendritic segment (**Yadav et al. 2012**).

Pyramidal neurons belonging to different cortical layers also exhibit different dynamics. For example, Tjia et al. found that apical dendrites of layer 2/3 neurons in mouse barrel and motor cortices have higher spine densities and turnover rates than corresponding apical dendrites of layer 5 cells (**Tjia et al. 2017**). Additionally, while layer 5 apical dendrites decrease in spine density throughout the animal's life, layer 2/3 apical dendrites maintain a stable spine density (**Ma et al. 2015, Tjia et al. 2017, Zuo et al. 2005**). Differences are also observed between cortical regions; at P28 in mice, basal rates of dendritic spine motility on layer 5 pyramidal neurons differ between visual, auditory, and somatosensory cortices, with the visual cortex exhibiting the least structural dynamics (**Majewska et al. 2006**).

Across neocortical areas, spines become more stable with age, with the majority of spines persisting at least 8 days by around two months of age (**Holtmaat et al. 2005, Majewska et al. 2006**). However, in hippocampal CA1, an archicortical area critical for episodic memory, dendritic spines exhibit much more turnover than in the neocortex (with models suggesting a near 100% turnover rate over sufficiently long time periods), commensurate with their role of the hippocampus in continuously encoding novel experiences (**Attardo et al. 2015**). Additionally, NMDA receptor blockade, which stabilizes spines in the neocortex, transiently increased the rate

of spine elimination in CA1 (**Attardo et al. 2015**). The differences between neocortex and CA1 suggests that spine dynamics are specialized for the functions of a given area.

#### *2.4.6. Dendritic spine imaging in vivo*

Until about two decades ago, insight about how neuronal structures change *in vivo* had to be extrapolated from single time-point observation in fixed tissues, or inferred from experiments done in culture. The advent of 2-photon scanning light microscopy (2PSLM) was an incredible boon to neuroscience as a field, as it allowed unprecedented high-resolution imaging of deep brain structures *in vivo* over time (**Denk et al. 1990, Kerr and Denk 2008, Svoboda et al. 1997**). This technique now allows us to finely dissect neural circuits by probing their subcellular components, as the properties of dendritic domains within a neuron, or even individual synapses, can now be examined *in vivo* with genetic and imaging techniques (**Chen et al, 2013**).

While the first reported instance of dendritic imaging *in vivo* used a Sindbis virus to express enhanced green fluorescent protein (eGFP) in layer 2/3 cells of the rat barrel cortex, most subsequent studies (including this dissertation) have taken advantage of the thymocyte differentiation antigen 1 (Thy1) transgenic mice (**Feng et al. 2000, Lendvai et al. 2000**). These mice express fluorescent reporters through the thy1 promoter, which results in the labeling of a sparse subset of neurons. This allows for fine subcellular structures, including dendritic spines, to be resolved *in vivo* (**Pan and Gan 2008**). As spine dynamics are a proxy for synapse dynamics, 2PSLM tracking of dendritic spines over periods of time *in vivo* allowed several groups to investigate synaptic changes in living animals for the first time, as detailed below.

#### *2.4.7. Dendritic spines as a substrate for plasticity*

Structurally, dendritic branches remain mostly stable in adulthood, other than small tip extensions and retractions (**Holtmaat and Svoboda 2009, Koleske 2013, Mizrahi and Katz 2003, Trachtenberg et al. 2002**). However, dendritic spines can remain highly dynamic, making them a prime substrate for plasticity (**Fu and Zuo 2011, Yu and Zuo 2011**). Indeed, spine formation and elimination, as well as spine size modulation, have been linked to the induction of LTP and LTD, respectively, one line of evidence implicating spine dynamics as a method of network modification and information storage in the nervous system (**Wiegert and Oertner 2013, Hasegawa et al. 2016, Zhou et al. 2004**).

Spine dynamics are modulated by experience, and various studies in sensory cortices have examined how different spine populations respond to environmental influence (**Figure 1.5**). In mice from P11-P13, unilateral whisker trimming decreases spine motility in layer 2/3 pyramidal cells of the contralateral barrel cortex but does not affect spine density, length, or shape (**Lendvai et al. 2000**). Later, in adolescence (1-3 months), unilaterally plucking all whiskers as a form of sensory deprivation reduces spine elimination and delays pruning in layer 5 cells of the contralateral barrel cortex (**Zuo et al. 2005**). This effect persists in adulthood (>4 months), though the magnitude becomes reduced and is only noted over longer time scales (on the order of weeks; **Zuo et al. 2005**).

Other studies induced sensory stimulation through enriched environments or unilateral chessboard plucking (plucking every other whisker to cause imbalance in the activation of neighboring whisker columns) and found increased spine turnover in barrel cortex (**Trachtenberg et al. 2002, Holtmaat et al. 2006, Yang et al. 2009**). Importantly, the effect of the environment on spine dynamics target specific neurons and layers, rather than the general

cortical region. Holtmaat et al. found that whisker trimming preferentially stabilized new spines while causing the elimination of previously persistent spines specifically on layer 5 pyramidal cells with complex apical tufts, and not those with simple tufts (**Holtmaat et al. 2006**). In adolescent (P30) mice, neonatal bilateral whisker trimming (from P0-7) decreases spine density in the apical tufts of both layer 2/3 and layer 5, but increases formation and elimination in layer 5 while decreasing formation with no significant change in elimination on layer 2/3 apical tufts (**Tjia et al. 2017**)

In the visual cortex, sensory experience also causes significant changes in spine dynamics. Mice that are raised in darkness from birth exhibit higher spine motility and less mature spine morphology compared with control, a phenotype which is rescued by a few days of light exposure during the critical period (P21-28; **Tropea et al. 2010**). This effect of light exposure can be partially mimicked by enhancing inhibitory responses through injections of diazepam, which decreases spine motility to control levels but does not increase spine maturation (**Tropea et al. 2010**). This, combined with previous data showing that EI balance is crucial for the correct development of visual function during the critical period (**Fagiolini and Hensch 2000**), suggests that the EI ratio is important in regulating spine maturation and plasticity during development, which are critical in effecting functional changes in the visual system.

Sensory deprivation also can affect spine dynamics in the visual system. In mice, monocular deprivation (MD) increases spine formation in the apical dendrites of layer 5 neurons in the binocular region of visual cortex, causing an increase in spine density (**Hofer et al. 2009**). When binocular vision is restored, spine formation returns to baseline levels, but the increased density persists and many spines induced by the MD are stabilized (**Hofer et al. 2009**). Importantly, spine formation did not increase when the eye was closed again later in life,

suggesting that spines formed by the original MD serve as a structural trace for the memory of the experience (**Hofer et al. 2009**).

In addition to their importance in responding to external cues, spines are a substrate for learning and memory. In the motor cortex in particular, spine dynamics change dramatically in response to motor learning (**Figure 1.5, Yu and Zuo 2011**). When mice are trained in a forelimb reaching task, within the first hour of the first training session, new spines form in apical dendrites of layer 5 pyramidal neurons contralateral to the trained arm (**Xu et al. 2009**). This is later followed by increased spine elimination, eventually resulting in a similar overall spine density to that in control mice. Similar increases in spine turnover are observed in mice performing other tasks such as rotarod and pasta handling tasks (**Xu et al. 2009, Yang et al. 2009**). Importantly, the spine elimination mostly affects spines that existed prior to motor training, and the newly-formed spines induced by learning are preferentially stabilized, lasting long after training ceases (>4 months; **Xu et al. 2009**). The degree of spine formation is also well-correlated with the degree of learning acquisition, and the survival of these learning-induced spines is associated with maintenance of the acquired motor skills, important results in correlating spine dynamics with learned behavior (**Xu et al. 2009**). In an elegant demonstration that the newly formed spines are specifically important in learning new tasks, Hayashi-Takagi et al. showed that selectively shrinking spines that were potentiated by motor learning disrupted the learning of the task (**Hayashi-Takagi et al. 2015**). Additionally, a study in songbirds showed that higher levels of spine turnover before song-learning correlates with a greater capacity for subsequent song imitation, suggesting that the potential for behavioral learning can be related to how “primed” sensorimotor circuits are based on their structural dynamics (**Roberts et al. 2010**).



In adult mice that were trained in a motor task in adolescence, re-training does not increase spine turnover, while training naive mice of the same age does, indicating that the neural circuitry involved in a learned motor task can persist throughout life (**Xu et al. 2009**). However, training pre-trained mice with a novel task does induce a robust increase in spine turnover, providing further evidence that different motor skills are encoded by different synapse populations in the motor cortex (**Xu et al. 2009**). Recapitulating the previously discussed result in visual cortex, spines induced by experiences can persist as structural traces of those experiences, allowing the brain to rapidly adapt to similar experiences in the future (**Hofer et al. 2009, Xu et al 2009**).

The spine dynamic changes induced by motor learning are, as for sensory experiences, layer-, neuron- and branch-specific. One group reported that training mice to perform a forelimb-specific motor learning task promotes spine turnover in the apical dendrites of layer 5 neurons, but not those of layer 2/3 (**Tjia et al. 2017**). However, a different group trained mice to perform a lever-press task and did note increased spine formation in the apical dendrites of layer 2/3 pyramidal neurons, though not in perisomatic dendrites in the same cells (**Chen et al. 2015**). These differences may be attributed to the different tasks performed, or perhaps other minute differences in experimental conditions, again showing the specificity of spine dynamic responses to specific circumstances. Interestingly, in the latter study, Chen et al. noted that compartment-specific spine changes are directly related to inhibitory input onto that compartment by specific populations of inhibitory interneurons (**Chen et al. 2015**).

In another study of motor learning's effect on spine dynamics, training rat in a skilled grasping task increases spine density and dendritic complexity specifically in the layer 5 pyramidal neurons involved in controlling the distal forelimb, despite pyramidal cells for

proximal and distal forelimb control being intermingled in the motor cortex (**Wang et al. 2010**). Additionally, Yang et al. found that sleep promotes dendritic spine formation on specific branches of layer 5 pyramidal neurons depending on the motor task learned (**Yang et al. 2014**).

Interestingly, Peters et al. note that the layer 5 network activity patterns of dissimilar movements become less correlated with motor learning (**Peters et al. 2017**). This is consistent with the increased elimination of pre-existing layer 5 spines seen in motor learning, and may reflect how spine dynamic changes lead to network rewiring and changes in the systemic response.

Importantly, the lack of obvious changes in spine dynamics for a given population of neurons in response to learning does not necessarily imply a lack of participation. For example, while layer 2/3 pyramidal neurons do not increase their spine dynamics in response to a motor learning task (**Tjia et al. 2017**), Peters et al. showed that there is convergence of layer 2/3 pyramidal cell activity as an animal perfects a motor behavior task (**Peters et al. 2014**). Additionally, Rioult-Pedotti et al. showed that motor learning occludes LTP between L2/3-L2/3 connections while enhancing LTD (**Rioult-Pedotti et al. 2000**). Taken together, these results suggest that motor learning involves a combination of new circuitry forming (spine dynamics increase) in some populations and synapse strength modulation in others. The combination of both mechanisms, compared to one or the other, can greatly increase the capacity for memory storage in the brain (**Chklovskii et al. 2004, Stepanyants et al. 2002, Stepanyants and Chklovskii 2004**).

Sensory cortices also can undergo changes in response to motor learning. In mice learning a whisker-dependent object localization task, layer 2/3 pyramidal cell dendrites located in layer 1 (a site known to be important for sensorimotor integration) show enhanced spine

growth before expert learning is achieved, the degree of which is correlated with task mastery (**Kuhlman et al. 2014**).

Spine dynamics have also been studied in association cortices. For example, dendritic spines in mouse frontal association cortex were found to be selectively eliminated following fear conditioning and formed following fear extinction (**Lai et al. 2012**). This is mechanistically interesting as one might expect spines to be formed as a structural trace for the fear memory, while extinction could selectively eliminate those spines. Instead, the formation of new spines accompanying extinction correlates well with the fact that re-exposure to fear conditioning results in more rapid freezing, suggesting that the animal did not forget the contextual fear, but rather learned not to be afraid after extinction training.

Finally, there is also evidence for experience-dependent changes in spine dynamics outside the neocortex. For example, spines in Hippocampal CA1 shows changes in their dynamics following exposure to an enriched environment (**Kitanishi et al. 2009**). Additionally, dopamine (the canonical “reward” signal) can modulate spine dynamics on medium spiny neurons in the striatum (**Yagishita et al. 2014**).

#### *2.4.8. Evolutionary differences in dendritic spine properties*

Several studies have quantitatively compared spine morphology, density, and developmental time course between humans, primates, and mice. In humans, dendritic spines exhibit higher density, longer necks, and larger heads than those in mice (**Benavides-Piccione et al. 2002**). Dendritic spine density in layer 3 pyramidal cells is also higher in humans than in marmoset and macaque monkeys across several neocortical areas (**Elston et al. 2001**). In addition, spinogenesis in prefrontal cortex displays neoteny in humans, reaching maximum spine density significantly later

in prefrontal cortex than in auditory cortex (age 15 months compared to 3 months); substantial reorganization also occurs in the prefrontal cortex until the third decade of life (**Huttenlocher and Dabholkar 1997, Petanjek et al. 2011**). These results show that evolution of synapse regulation in humans was not uniform across the cortex, and suggests that the emergence of human-like phenotypes arose from targeted evolution of specific areas.

#### *2.4.9. Dendritic spines in brain disorders*

Changes in dendritic spine morphology and dynamics have been implicated in a variety of distinct disorders (**Fiala et al. 2002, Fu and Ip 2017, Penzes et al. 2011, Sala and Segal 2014**). In neuropsychiatric disorders, spine abnormalities are often associated with behavioral deficiencies and cognitive decline (**Fiala et al. 2002, Penzes et al. 2011**). For example, in a mouse model of Rett Syndrome, both spine formation and elimination are increased; however, new spines are more vulnerable to elimination than in wild-type mice, which results in a net loss of spines (**Jiang et al. 2013**). In a different neuropsychiatric disorder, Fragile X mental retardation, mouse models exhibit increased spine density in turnover along with a delay in spine maturation, and there is less plasticity seen in response to sensory manipulation (**Comery et al. 1997, Cruz-Martin et al. 2010, Irwin et al. 2000, Padmashri et al. 2013, Pan et al. 2010**). Additionally, in schizophrenia, there is a selective loss of smaller spines (**MacDonald et al. 2017**). Spine pathology is also seen in several autism-related disorders, where the number and morphology of spines are altered (**Phillips et al. 2014**).

Spine aberrations are also seen in models of ischemia. Rapid spine loss occurs following severe ischemia, which is reversible if the area is reperfused within a short period of time (20-60

minutes; **Zhang et al. 2005**). Additionally, spine turnover increases in the peri-infarct area, while distant areas are unaffected (**Brown et al. 2009, Johnston et al. 2013**).

Spine irregularities also occur in models of pain. Increased spine turnover is seen in a mouse model of chronic pain, with an initial elevation of spine formation followed by an increase of spine elimination, causing a spike in spine density followed by pruning (**Kim and Nabekura 2011**). This change in spine turnover is prevented by TTX blockade, suggesting that post-lesion spine remodeling is activity-dependent (**Kim and Nabekura 2011**).

Finally, spine properties are affected in various neurodegenerative diseases. In Alzheimer's Disease, spine loss is accelerated near  $\beta$ -amyloid plaques in the cortex (**Tsai et al. 2004, Spires et al. 2005**). In mouse models of Huntington's disease, there is an increase of spine formation; however, newly formed spines are eliminated and not incorporated into local circuits, which results in a net decrease in spine density (**Murmu et al. 2013**).

### **3. SRGAP2**

Humans evolutionarily split from our closest relatives, chimpanzees and bonobos, 6-7 million years ago (**Tyler-Smith and Xue 2012**). Since then, human cognitive abilities have wildly diverged from that of non-human primates, attributed to our larger and more complex brains. Gene duplication is recognized as perhaps the main driver of this evolutionary innovation, as duplicated genes are likely to be less constrained than the ancestral gene (due to their natural redundancy), and therefore prone to acquire novel functions. Therefore, such genes are prime sources for adaptive evolution, and many recent efforts are focused on determining how such duplications alter gene function to promote a more "human" phenotype (**Lynch and Katju 2004, Dennis et al. 2012**).

In particular, the human and great-ape lineages have exhibited a surge of genomic duplications throughout the last 10 million years of evolution (**Marques-Bonet 2009**), and genes involved in neurodevelopment have specifically experienced many duplication events in humans (**Fortna et al. 2004, Goidts et al. 2006**). Indeed, defects with many of these highly polymorphic genes are associated with intellectual disability, autism, schizophrenia, and epilepsy (**Antonacci et al. 2009**). About ~49% of the gene duplicates are largely invariant in copy number among humans, suggesting that their function has become critical through evolution (**Sudmant et al. 2010**).

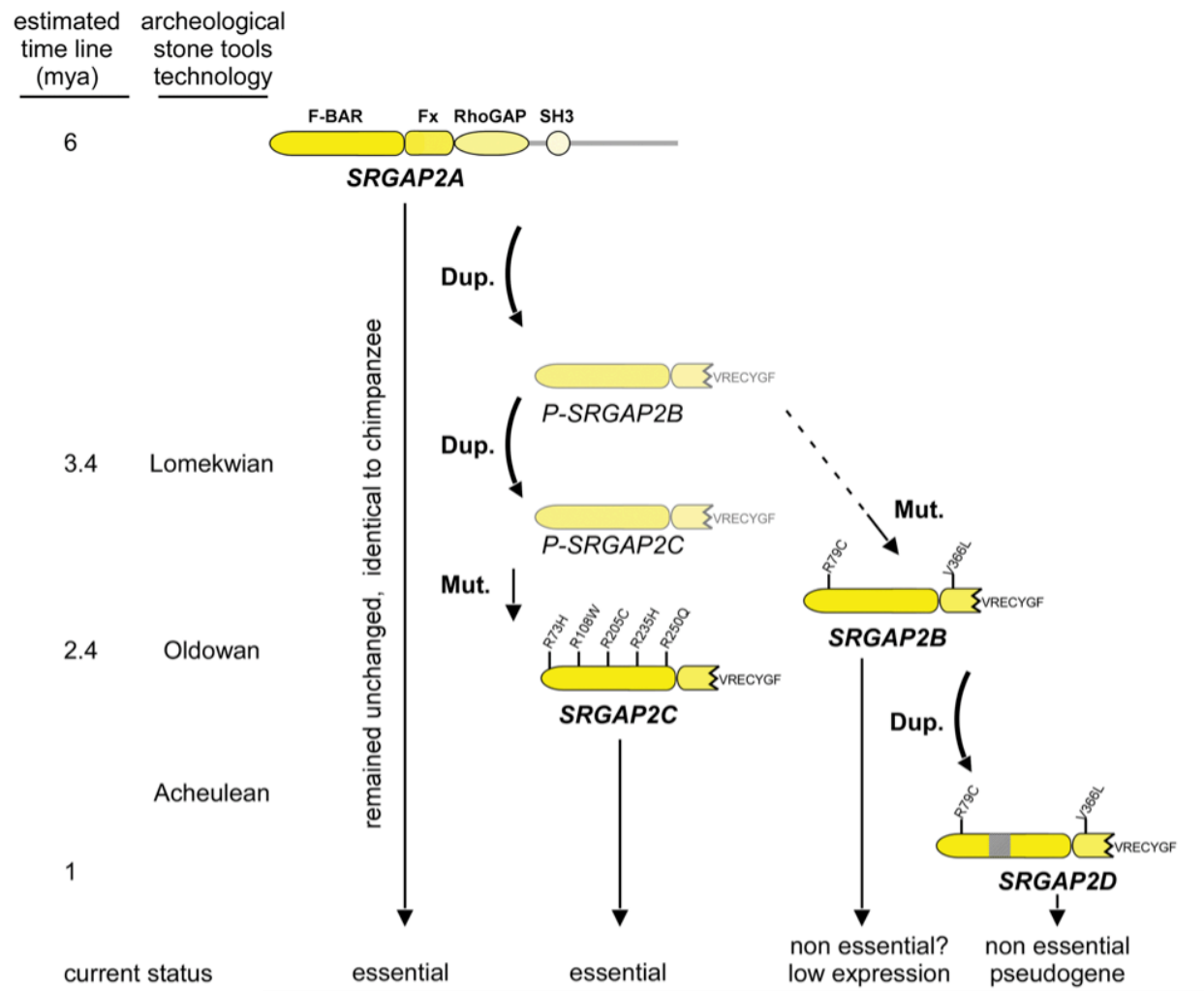
In 2010, in an effort to identify human-specific gene expansions associated with brain development, Sudmant et al. identified 23 genes duplicated in the *Homo* lineage after our evolutionary split from other primates (**Sudmant et al. 2010**). Of these genes, SRGAP2 (slit-robo GTPase activating protein 2) has several known functions in cortical and synaptic development, providing an avenue of investigation into how human brains diverged from non-human primates and other mammals.

### ***3.1. SRGAP2 structure and biochemical interactions***

SRGAP (slit-robo GTPase activating protein) proteins are named for the ability of their C-terminal SH3 (SRC Homology 3) domain to bind the Robo family of receptors, each of which are involved in neuronal development and regulation (**Lucas and Hardin 2017, Wong et al. 2011**). In vertebrates, there are 4 SRGAPs (1-4); SRGAP1-3 share 60-80% homology, while SRGAP4 is the least conserved at 51% identical to SRGAP3 (**Lucas and Hardin 2017**).

SRGAP2 is also known as formin-binding protein 2 (FNBP2) or ARHGAP14 (Rho GTPase-activating protein 14). In mice, the SRGAP2 protein is built from 1,045 amino acids and

contains an F-BAR (Fes-Cip4-Bin/Amphiphysin/Rvs, also known as EFC) domain at the terminus, followed by a RhoGAP and a SH3 domain (**Figure 1.6; Sporny et al. 2017, Guerrier et al. 2009**). This general architecture is consistent across the 4 SRGAP proteins (**Lucas and Hardin 2017**).



**Figure 1.6 - Evolutionary history and domain organization of SRGAP2**

Evolutionary history diagram detailing the duplication (Dup.) and mutagenesis (Mut.) events in human SRGAP2 genes and their current status in modern-day humans. A genetic approximate timeline and parallel stone tools technology archaeological dating (**Harmand et al. 2015**) shows correlation between the first and second mutagenesis events with the first and second generations of stone tools, ~3.3 and ~2.4 Ma, respectively. The missing carboxy-segment of the Fx in all the duplicated copies is indicated as a zig-zag tear followed by a seven-residue (VRECYGF) carboxy-terminal addition. Also indicated and detailed are the nonsynonymous mutations in the

modern duplicated proteins. The primal forms of SRGAP2B and SRGAP2C, which existed prior to the accumulation of mutations are named with the prefix “P-.” Figure from **Sporny et al. 2017**

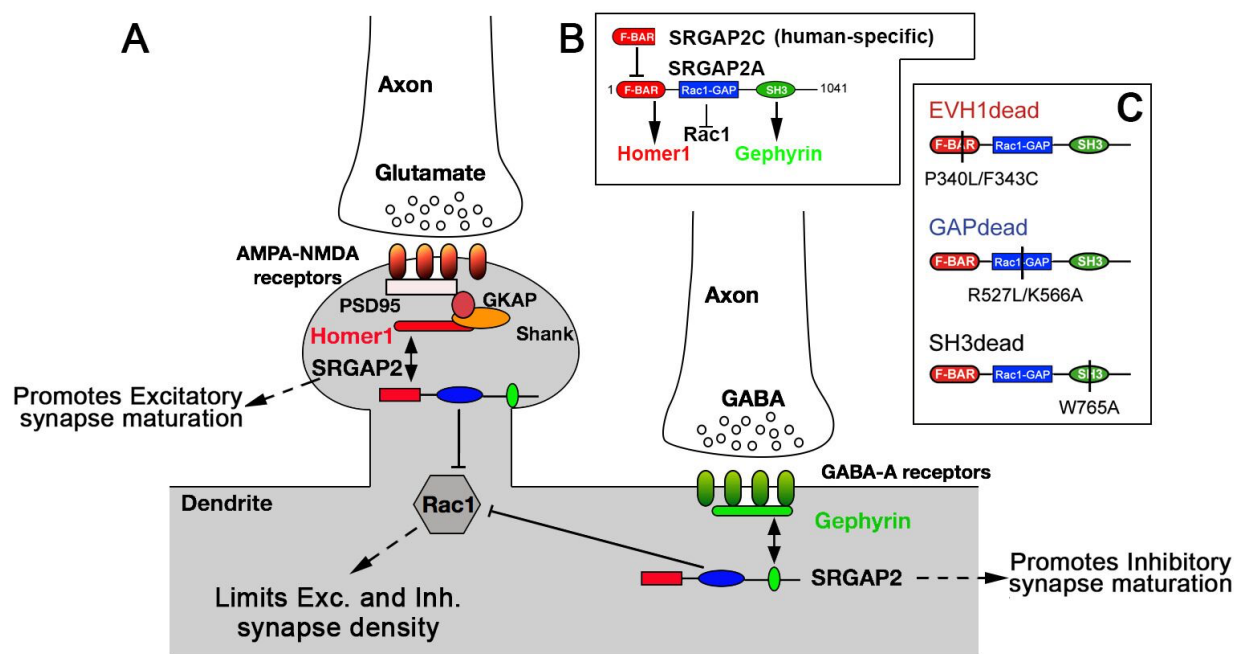
The BAR domain superfamily contains three main groups, including F-BARs; these are composed of a series of alpha-helices and forms a dimerization motif. These assemble into anti-parallel dimers that interact with membranes through their “N-surface,” which can have either concave, convex, or flat conformations. In particular, F-BAR homodimers can contort into a quaternary “banana-like” structure, and (like other BAR domains) exhibit the ability to bind and deform membranes both *in vitro* and in living cells. This membrane binding is facilitated by electrostatic interactions between the positively charged residues on the surface of the protein and the negatively charged membrane phospholipids (**Guerrier et al. 2009, Sporny et al. 2017**).

Canonically, BAR, and F-BAR domains possess concave N-surfaces which associate with cellular membrane invaginations, while I-BARs associate membrane protrusions through their convex N-surfaces. Surprisingly, however, while other F-BAR domains-containing proteins (e.g., FBP17, Syndapin, FCHo2) are mostly characterized by their ability to induce membrane tubulations and invaginations (**Frost et al. 2008, Habermann 2004, Henne et al. 2007, Itoh and De Camilli 2006, Kakimoto et al. 2006, Kessels and Qualmann 2015, Peter et al. 2004, Shimada et al. 2007, Sporny et al. 2017**), the F-BAR domain of SRGAP2 localizes it to cellular protrusions like dendritic spine heads and lamellipodia, and has the ability to form filopodia-like membrane protrusions (**Guerrier et al. 2009**).

Recently, Sporny et al. showed that in SRGAP2’s F-BAR domain has some alpha helices bent in the opposite direction from other F-BAR-containing proteins (where these alpha helices are bent toward the N-surface). This generates a convex membrane binding surface instead of the canonical concave surface. This results in SRGAP2’s F-BAR domain more closely resembling



the quaternary structure and charge distribution of inverse-BAR (I-BAR) domains from proteins such as IRSp53 or MIM, which are known to induce filopodia through the curvature of I-BAR homodimers (Lim et al. 2008, Mattila et al. 2007, Millard et al. 2007, Saarikangas et al. 2009, Sporny et al. 2017). Similarly, other F-BAR domain-containing proteins have been shown to induce filopodia, likely through the same structural mechanism (Chitu et al. 2005, She et al. 2002).



**Figure 1.7 - The role of SRGAP2 in synaptic regulation**

**A.** SRGAP2 localizes to dendritic spines and interacts with regulators of dendritic spine dynamics. **B.** Human SRGAP2C is a truncated paralog of mouse SRGAP2 and inhibits human SRGAP2A. **C.** Mutated versions of SRGAP2 used for loss-of-function experiments in Fossati et al. 2016. Figure from the Polleux lab

Recent investigations showed that the F-BAR domain of SRGAP2 binds Homer1c through an EVH1 binding domain (Figure 1.7, Fossati et al. 2016). Homer1 assists in the assembly of excitatory postsynaptic scaffoldings by binding Shank and recruiting the Shank-

GKAP-PSD-95 complex into spines to stabilize AMPA and NMDA glutamate receptors at the postsynaptic density (**Hayashi et al. 2009, Sheng and Hoogenraad 2007, Tu et al. 1999**). Homer1c also regulates spine signaling through interactions with mGluR5, Ip3, and Ryanodine receptors (**Bockaert et al. 2010, Ting et al. 2012**).

Each SRGAP protein also contains a RhoGAP and regulates RhoGTPase family members, affecting cytoskeletal dynamics during a plethora of processes in the nervous system and elsewhere (**Lucas and Hardin 2017**). SRGAP2 specifically stimulates the GTPase activity of Rac1, a function which is likely autoinhibited at rest, similar to many other RhoGEFs/RhoGAPs and other BAR domain containing proteins (**Figure 1.7, Eberth et al. 2009, Guerrier et al. 2009, Ma et al. 2013, Mason et al. 2011, Rossman et al. 2005**). Rac1 is a protein implicated in several processes regulating neuronal development (**Govek et al. 2005**). In particular, it is involved in regulating radial migration and neurite outgrowth (**Causeret et al 2008, Govek et al. 2005, Kawauchi et al. 2003, Konno et al. 2005, Yoshizawa et al. 2005**). Thus, the GAP activity of SRGAP2 may inactivate Rac1, locally increasing Cdc42 activity and activating pathways that push F-actin into filopodia formation (**Raftopoulou and Hall 2004**). Alternatively or in addition, Rac1 inactivation can cause increased Rho1 activity (as Rac1 inactivates RhoA), which could cause activation of the formin mDia2 and increase actin nucleation (**Nimnual et al. 2003**).

Finally, the SH3 domain of SRGAP2 is a feature it shares with a high proportion of F-BAR domain-containing proteins (**Itoh and De Camilli 2006**). SH3 domains are protein-protein interaction domains which can recruit various signaling proteins, such as regulators of endocytosis and actin dynamics, cell migration, and cell adhesion (**Aspenstrom et al. 2006, Chitu et al. 2005, Itoh and De Camilli 2006, Lucas and Hardin 2017**). Specifically, SH3

domain of SRGAP2 binds the Robo1 receptor as well as N-WASP, though the functional relevance is unknown (**Linkermann et al. 2009, Wong et al. 2001**). Additionally, SRGAP2 interacts with the formin FMNL1 through its SH3 domain, both directly and indirectly inhibiting its ability to sever F-actin (**Mason et al. 2011**). More recently, Gephyrin, a critical component of the postsynaptic scaffolding of inhibitory synapse (see above section on inhibitory synapses), was identified as a partner for SRGAP2 at through its SH3 domain (**Figure 1.7, Okada et al. 2011**). Both the RhoGAP and SH3 domains are also involved in SRGAP2's ability to dimerize (**Sporny et al. 2017**).

The function of SRGAP2 results from the synergy of its component domains. For example, activation of SRGAP2 can begin with its recruitment to plasma membrane by one of its target proteins at the SH3 domain (e.g., Robo1; **Wong et al. 2001**). Here, it can directly bind the plasma membrane through its F-BAR domain at sites of protruding curvatures, directing its action to specific subcellular compartments like dendritic spines (**Guerrier et al. 2009, Coutinho-Budd et al. 2012, Yamazaki et al. 2013**). Here, its RhoGAP domain can inactivate local pools of Rac1 and CDC42, causing the breakdown of the local actin cytoskeleton and retraction of membrane protrusions (**Wong et al. 2001, Fritz et al. 2015**).

### ***3.2. SRGAP2 expression***

As is fitting with their roles in neuronal development, all SRGAP family proteins are prevalent in neural tissues (**Bacon et al. 2009, Chen et al. 2012, Waltereit et al. 2008, Wong et al. 2001**). SRGAP2 in particular is expressed through zones of neuronal proliferation (ventricular and subventricular zones), the cortex, various thalamic nuclei, the hippocampus (though not the dentate gyrus), and other brain areas (**Bacon et al. 2009, Guerrier et al. 2009**).

Temporally, SRGAP2 is expressed throughout the period of nervous system development starting at E11.5, and is upregulated at the end of cortical inhibition, with its peak corresponds with that of neuronal migration in the cortex (P1; **Bacon et al. 2009, Guerrier et al. 2009**). It is primarily expressed in neocortex during this developmental periods of high synaptogenesis, though it persists at a reduced level later on (**Charrier et al. 2012, Guerrier et al. 2009**).

In the cortex, SRGAP2 is expressed throughout layers 2-5, with weak expression in layer 6 (**Bacon et al. 2009**). Subcellularly in culture, SRGAP2 is found at cell peripheries and often localizes to F-actin-rich membrane protrusions and excitatory synapses (**Charrier et al. 2012, Guerrier et al. 2009**).

Interestingly, the temporal pattern of SRGAP2 expression is not uniform in the central nervous system. For example, in the hippocampus, SRGAP2 is expressed weakly at P1 in CA1 with a stronger presence in CA3; this expression pattern reverses by P7 (**Bacon et al. 2009**).

### ***3.3. Function of SRGAP2***

SRGAP family proteins each function to link specific plasma membrane regions to actin cytoskeleton remodeling, regulating a myriad of processes in neuronal migration, axon guidance/branching, and dendritic spine maturation (**Lucas and Hardin 2017, Sporny et al. 2017**).

#### ***3.3.1. Cortical migration and development***

In mice, commensurate with its presence in the developing cortex, SRGAP2 has several functions in regulating cortical migration/development/morphogenesis and spine mobility. Specifically, SRGAP2 limits the rate of cortical migration and promotes the complexity and branching of the leading process (LP); knockdown of SRGAP2 increases the rate of radial

migration in the cortex and reduces LP complexity/branching (**Charrier et al. 2012, Guerrier et al. 2009, Guo and Bao 2010**).

Knockdown of SRGAP2 increases the rate of cortical migration and reduces LP (leading process) complexity/branching (**Guerrier et al. 2009**). This is fitting with the ability of its F-BAR domain to interact with lamellipodia and induce membrane protrusions, allowing for a more complicated cytoskeletal architecture at the LP and perhaps more exploratory behavior (see above).

Somewhat surprisingly, a lack of SRGAP2 does not disrupt neurite initiation in cortical neurons, though it can promote initiation and branching (**Guerrier et al. 2009**). This is likely due to compensation from other proteins involved in filopodia formation which are required for neurite initiation (**Dent et al. 2007**).

### 3.3.2. *Excitatory synapses*

Along with its impact on cortical migration, SRGAP2 is an important regulator of excitatory synapse development, associating with the postsynaptic density to promote spine maturation and limit spine density *in vivo* (**Figure 1.7, Charrier et al., 2012**). SRGAP2 knockdown in mice decreases the size of dendritic spines and their GluA2-containing receptor clusters at juvenile (~P20) ages, while increasing the density of spines by ~50% and increasing the length of spine necks (**Charrier et al. 2012, Fossati et al. 2016**). The decrease in spine size does not persist throughout maturation, and indeed at young adult stages (~P65) spine maturation measured by head size is almost indistinguishable between SRGAP knockdown mice compared to control mice; however, these spines show a persistent increase in neck length and density (**Charrier et**

**al. 2012**). These results suggest that SRGAP2 promotes spine spine maturation while limiting spine density and neck length.

The ability of SRGAP2 to affect spine density and neck length is dependent on the activity of its Rac1-GAP domain. However, regulation of spine head size (and by proxy, synaptic strength) depends on its ability to bind Homer1 but does not depend on either the Rac1-GAP nor the SH3 domain, despite the putative role of the SH3 domain in binding actin-regulating proteins and thereby controlling spine morphology (**Fossati et al. 2016**).

With the importance of its binding partners, SRGAP2 may function by promoting local recruitment of scaffolding proteins (e.g. Gephyrin, Shank, PSD-95) to nascent synapses by interacting with highly-curved membrane structures like filopodia or dendritic spines through the curved quaternary structure of its F-BAR domain (see above; **Fossati et al. 2016, Sporny et al. 2017**). SRGAP2 may also couple these scaffolding structures with other proteins involved in synaptic adhesion or organization, or induce conformational changes in its partners leading to synapse assembly. Importantly, SRGAP2 promotes these processes through cell-autonomous developmental mechanisms, rather than through homeostatic or adaptive responses.

Additionally, SRGAP2 could be linked to regulation of neocortical synaptic density, functional plasticity, and critical periods through its Rac1-GAP activity (**Figure 1.7**). This limits spine density and restricts compartmentalization of both excitatory and inhibitory synapses by modulating the length of spine necks, and also controls the occurrence of spine-localized inhibitory synapses (**Alvarez and Sabatini 2007, Cahill et al. 2009, Cerri et al. 2011, Chiu 2013, Luo et al. 1996, Yuste 2011**).

### 3.3.3. Inhibitory synapses

More recently, **Fossati et al.** found that SRGAP2 also regulates inhibitory synapses in a similar manner to excitatory synapses by examining the density and size of inhibitory synapses of oblique apical dendrites of layer 2/3 pyramidal neurons of somatosensory cortex.

In juvenile (~P21) mice, SRGAP2 knockdown increased Gephyrin cluster density by ~75%, while decreasing cluster size ~20%. This is accompanied by a decrease in size of GABA-A receptor clusters (visualized through a  $\gamma$ 2-GABA-A receptor-pHluorin construct). Additionally, SRGAP2 knockdown affected the distribution of inhibitory synapses within neurons, increasing the proportion of gephyrin clusters located in dendritic spines by ~50%. Gephyrin cluster growth also extended to young adult (~P65) ages, while in WT mice, Gephyrin cluster size was essentially fixed by P21. Young adult mice also maintained higher density of Gephyrin clusters, along with the enrichment of Gephyrin clusters within dendritic spines (~40% compared to ~25% in control; **Fossati et al. 2016**). These findings recapitulate the effect of SRGAP2 knockdown for excitatory synapses (see above, **Charrier et al. 2012**).

The effects of SRGAP2 on the density of inhibitory synapses requires its Rac1-GAP activity; however, the rate of maturation does not. Conversely, the ability of the SH3 domain to bind Gephyrin is required for inhibitory synapse maturation, but not synapse density. Additionally, the increase of Gephyrin clusters targeted to dendritic spine heads increases with Gephyrin cluster density, independent from cluster size. Therefore, SRGAP2's control over the density and subcellular distribution of inhibitory synapses appears to depend on its Rac1-GAP domain, while its ability to promote maturation and synapse size depends on direct binding of SRGAP2 to Gephyrin through its SH3 domain. Comparing these findings to those of excitatory synapses suggests that the Rac1-GAP domain functions as a master regulator for the density of

both excitatory and inhibitory synapses, while maturation and synaptic strength is controlled by separate domains (**Figure 1.7**). Interestingly, dysfunction of the F-BAR domain does not affect Gephyrin cluster density, size, or distribution, suggesting that SRGAP2-dependent regulation of excitatory synapses does not lead to indirect/homeostatic responses in the development of inhibitory synapses (**Fossati et al. 2016**).

Canonically, the assembly of excitatory and inhibitory synapses involve nearly mutually exclusive sets of proteins, with few genes known to regulate both types of synapses (**Fossati et al. 2016**). However, with the conservation and stringency of the E/I ratio throughout mammalian life, mechanisms must exist to coordinate the rate of maturation for both types of synapses. The ability of SRGAP2 to affect the density, localization, and maturation of both excitatory and inhibitory synapses might position it as an important regulator of cortical activity in general, which is reflected by its conservation throughout mammalian evolution and its implication in disorders when defective (see below).

Importantly, because the decrease in synapse size observed with SRGAP2 partial knockout or shRNA-mediated knockdown is accompanied by a homogeneous decrease in local postsynaptic markers like Homer1, PSD-95, NMDA/AMPA receptors, Gephyrin, and GABA-A receptors, it seems that SRGAP2 manipulation alters the rate of assembly of postsynaptic scaffolds without altering the general principle of synapse development (**Fossati et al. 2016**). With SRGAP2 knockdown, the E/I ratio is maintained with no differences seen in mEPSC/mIPSC amplitudes, though there is a mild decrease in the frequency of both, consistent with delayed maturation of postsynaptic scaffolding and the increased compartmentalization of synaptic input expected from the synaptic changes (**Araya et al. 2014, Fossati et al. 2016**).



### **3.4. *SRGAP2B-2D - Human-specific paralogs of SRGAP2A***

In non-human primates and other mammals, SRGAP2 exists as a single copy. SRGAP2 has been remarkably conserved over mammalian evolution, with the human ancestral copy remaining unchanged for at least the past 6 million years of evolution (**Sporny et al. 2017**). Humans are the only evolutionary lineage where gene duplications have affected SRGAP2 (**Figure 1.6, Charrier et al. 2012, Dennis et al. 2012**).

Dennis et al. investigated the 3 human-specific duplicates by leveraging haploid hydatidiform moles in an innovative technique, and found that duplication events occurred ~3.4, ~2.4, and ~1.0 million years ago. The first duplication generated SRGAP2B from SRGAP2A (the ancestral copy shared among all mammals), and duplicated a 258 kbp segment from chromosome 1q32.1 to 1q21.1. This first duplication event was incomplete, resulting in only 9 out of 22 exons and truncating the SRGAP2 protein in its F-BAR domain. Subsequent secondary duplications of >515 kbp on chromosome 1q21.1 to 1p12 and 1q21.1 generated SRGAP2C and 2D (SRGAP2C is similar to SRGAP2B, while SRGAP2D is more truncated). SRGAP2A retains functional equivalence to the ancestral SRGAP2 gene. The timing of SRGAP2B and 2C duplication corresponds well to the emergence of the *Homo* lineage from *Australopithecus* 2-3 million years ago, which is associated anatomically with the expansion of neocortex and the concomitant advent of stone tool usage, representing a dramatic change in behavior and culture (**Figure 1.5A, Dennis et al. 2012, Harmand et al. 2015**).

#### **3.4.1. *SRGAP2C - an inhibitor of SRGAP2A***

In the course of evolution, SRGAP2C has emerged as the dominant duplicate copy; while SRGAP2B and 2D both show evidence of transcription, both genes are highly polymorphic and

may represent pseudogenes (**Dennis et al. 2012**). SRGAP2C likely rendered SRGAP2B redundant with its duplication, and is the least polymorphic of all human-specific duplicate genes despite its position in a chromosomal region often vulnerable to nonallelic homologous recombination (**Dennis et al. 2012**). Similarly, SRGAP2A (the ancestral copy of SRGAP2) is largely stable, with large deletions observed only among children with developmental delay (**Cooper et al. 2011**, see below).

Despite being truncated, SRGAP2C retains the ability to dimerize with SRGAP2A, as the F-BAR domain of these proteins functions as a strong homodimerization domain; this allows SRGAP2C to function as an inhibitor for SRGAP2A (**Figure 1.7B**). Indeed, despite the variety of functions of SRGAP2A associated with its different domains, SRGAP2C antagonizes every known function of the ancestral gene (**Charrier et al. 2012, Fossati et al. 2016**). This may occur through interference with partner interactions through steric hindrance/direct competitions, prevention of proper targeting of SRGAP2A to synapses, or by modifying tertiary/quaternary structure, as with other proteins containing F-BAR and SH3 domains (**Fossati et al. 2016, Rao et al. 2010**). Recently, Sporny et al. demonstrated that SRGAP2C forms stable hetero-dimers with SRGAP2A, rendering it insoluble and unable to properly scaffold membranes, while also reducing its affinity to Robo1 (**Sporny et al. 2017**).

Interestingly, SRGAP2C accumulated several mutations since its duplication to acquire its full dominant negative function. Immediately upon its duplication, the truncated F-BAR domain already rendered it insoluble; however, later mutations facilitated tighter heterodimers with SRGAP2A, lending more support to its role as an evolutionary driver for human-specific traits (**Sporny et al. 2017**).

By antagonizing SRGAP2A, SRGAP2C induces the emergence of “human” traits in pyramidal neurons (**Benavides-Piccione et al. 2002, Elston et al. 2001, Huttenlocher and Dabholkar 1997, Petanjek et al. 2011**) . For example, neoteny is seen through delayed spine/gephyrin cluster maturation, and eventually there is higher morphological complexity in dendritic spines due to increased density and longer spine necks (**Charrier et al. 2012, Fossati et al. 2016**). This increase in the size and density of dendritic spines/gephyrin clusters matches the increased spine density, larger head size, and longer necks seen in humans (**Benavides-Piccione et al. 2002, Elston et al. 2001**), and the developmental delay recapitulates the prolonged processes of dendritic and synaptic maturation and synaptic elimination seen in humans relative to other mammals and non-human primates (**Bianchi et al. 2013, Huttenlocher et al. 1982**).

The delay in cortical migration and increased LP branching caused by SRGAP2C may lead to a longer period of radial migration, allowing neurons to journey over a longer distance and generate the thicker cortical wall seen in humans compared to non-human primates and rodents (**Dehay and Kennedy 2007, Rakic 2009, Sidman and Rakic 1973**). Indeed, the notion that slower/extended developmental processes may cause increasingly complex morphogenesis is supported by evidence showing genes involved in neoteny/heterochrony causing an expansion of neocortical surfaces (**Lui et al. 2011, Rakic 2009**). The confluence of SRGAP2C’s effects in synaptic development and cortical migration may have permitted the emergence of human cognitive abilities beyond those of non-human primates during evolution (**Defelipe 2011, Geschwind and Rakic 2013**).

As discussed above, disrupting the E/I ratio can result in a variety of problems in brain function. As SRGAP2C evolved became fixed in humans recently, without changing the E/I

ratio, mechanisms must exist to support the coordinated evolution of excitatory and inhibitory synapse development while preserving the E/I ratio.

While the nature of SRGAP2C's duplication, with its regulatory elements, implies that its expression is largely consistent with that of the ancestral SRGAP2A gene, how the two genes differ in their expression patterns is unclear (**Dennis et al. 2012**). While the mRNA of SRGAP2A and SRGAP2C are largely co-expressed (**Charrier et al. 2012, Polleux lab, unpublished observations**), differential expression of the protein products could be achieved through post-transcriptional mechanisms such as differences in subcellular mRNA trafficking or mRNA stability, as the main differences in the mRNA products of the two genes lie in the 3'-UTR.

It is possible that different spatiotemporal regulation of SRGAP2A and 2C may contribute to the higher levels of neurogenesis that are seen in human prefrontal neocortical areas (**Geschwind and Rakic 2013**). Such differences in the modulation of the two genes may selectively increase periods of developmental plasticity (i.e. critical periods), exemplifying the role of environmental stimulation in the evolution and development of human cortical circuits (**Geschwind and Rakic 2013, Varki et al. 2008**).

Thus far, studies on SRGAP2 have focused on its effect in early development and in young adults (P<75). Additionally, there have been no longitudinal studies on SRGAP2's effect on structural plasticity *in vivo*. It is therefore unclear how and whether SRGAP2 plays a role in regulating the formation and elimination of spines. In this dissertation, we model human-like spine dynamics in adult mice brain through SRGAP2 knockdown, and use this to study possible neural circuit changes that occurred through evolution. Through this, we can infer how changes

in dendritic structure/architecture leads to system-level differences between species. In Chapter 3, I investigate the role of SRGAP2 in modulating spine dynamics in adult barrel cortex. In Chapter 4, I examine how SRGAP2 affects the spatial distribution of newly formed spines.

## **CHAPTER 2**

### Methods

## Mouse lines

All mouse lines were maintained in standard conditions in accordance with guidelines established by the National Institute of Health and by the Institutional Animal Care and Use Committee. The Thy1-YFP H strain was B6.Cg-Tg(Thy1-YFPH)2Jrs/J (**Feng et al. 2000**), obtained from Jackson Laboratory. Genotyping was performed according to Jackson Laboratory recommendation. The SRGAP2 knock-out strain was a gene trapped (GT) allele of *Srgap2* (B6;129P2-*Srgap2*<sup>Gt(XH102)Byg/Mmc</sup>) obtained from Mutant Mouse Regional Resource Centers (MMRRC). Only heterozygous animals were used. Genotype was performed as described previously (**Charrier et al. 2012**). Mice were age P154-P171 at the start of imaging.

## Barrel cortex window and headpost implant

Mice were surgically implanted with an imaging window positioned over the left barrel cortex in combination with a stainless-steel headpost (described previously, **Kaifosh et al. 2013; Lovett-Barron et al. 2014**) for head fixation during *in vivo* imaging experiments. The imaging window/cannula was constructed by adhering (using Narland optical adhesive, cured by UV light) a 3.0 mm glass coverslip (64-0720, Warner) to a cylindrical steel cannula (3.0 mm diameter x 1.5 mm height). The surgical procedure was similar to that described previously (**Kaifosh et al. 2013; Lovett-Barron et al. 2014**), except cortex remained unaspirated, and care was taken to not disturb the dura when the skull fragment was removed. The implant coordinates were 1.3 mm posterior and 3.4 mm lateral to bregma. Following induction of anesthesia (Isoflurane: 3% induction, 1.0-1.5% maintenance, 1.0 L/min O<sub>2</sub>), administration of analgesia (buprenorphine 0.05-0.1 mg/kg, subcutaneous), the scalp was removed, and a 3.0 mm diameter craniotomy was performed with a fine-tipped dental drill (V00033, Henry-Schein). We then gently fit the cannula

into the craniotomy and affixed the headpost to the skull using dental cement (675572, Dentsply). Mice were kept warm post-surgery on a heating blanket and were active 15 minutes post-surgery. Analgesia (buprenorphine, 0.05-0.1 g/kg, subcutaneous) was administered every 12 hours postoperatively for 3 days.

### ***In vivo 2-photon imaging***

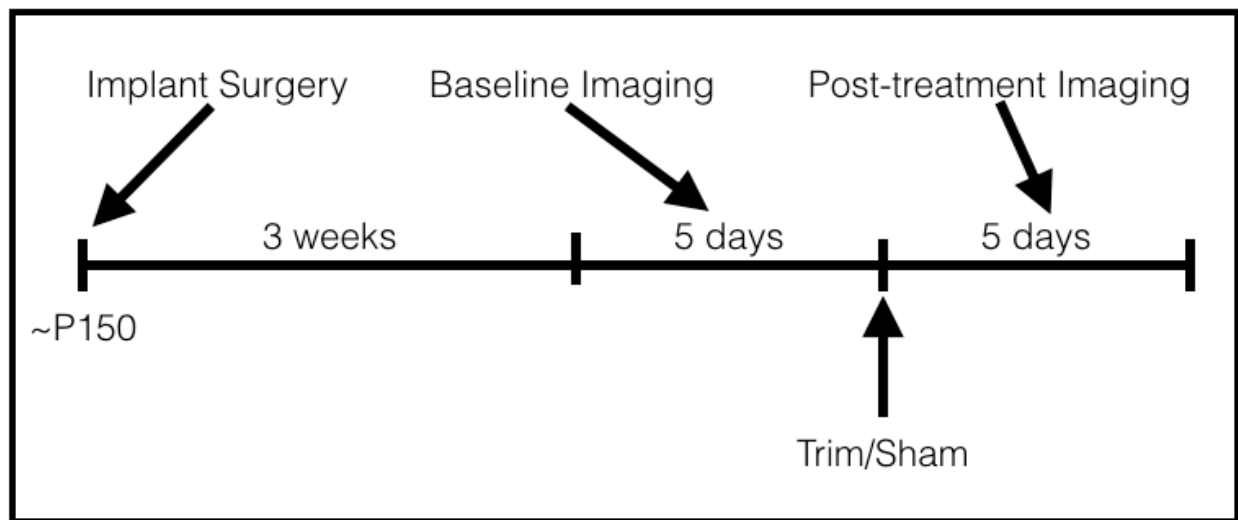
The imaging system was previously described, with the addition of an 8 kHz resonant galvanometer (Bruker). A piezoelectric crystal was coupled to the objective (Nikon 40x NIR water-immersion objective, 0.8 numerical aperture, 3.5 mm working distance, through distilled (milliQ) water) to allow for rapid displacement of the imaging plane in the z-dimension. Approximately 50-100 mW of laser power was used during imaging, with adjustments in power levels to accommodate variations in window clarity between animals. To optimize light transmission, we adjusted the angle of the mouse's head using two goniometers (Edmund Optics, +/- 15 degree range) such that the imaging window was parallel to the objective. We continuously acquired YFP signal through an emission cube set (HQ525/70m-2p; 575dcxr, Chroma technology) at 1024 x 1024 or 512 x 512 pixels, covering areas ranging from 37.5x37.5 um to 300 um x 300 um at 15 to 30 hz using a GaAsP photomultiplier tube (Hamamatsu Model 7422P-40). A custom dual stage preamp ( $1.4 \times 10^5$  dB, Bruker) amplified signals prior to digitization.

### **Image acquisition and processing**

2 weeks after surgery, mice were acclimated to the imaging apparatus by headfixed in the microscope for 20 minutes/day. 3 weeks post-surgery, a 300x300x350  $\mu\text{m}$  section of barrel



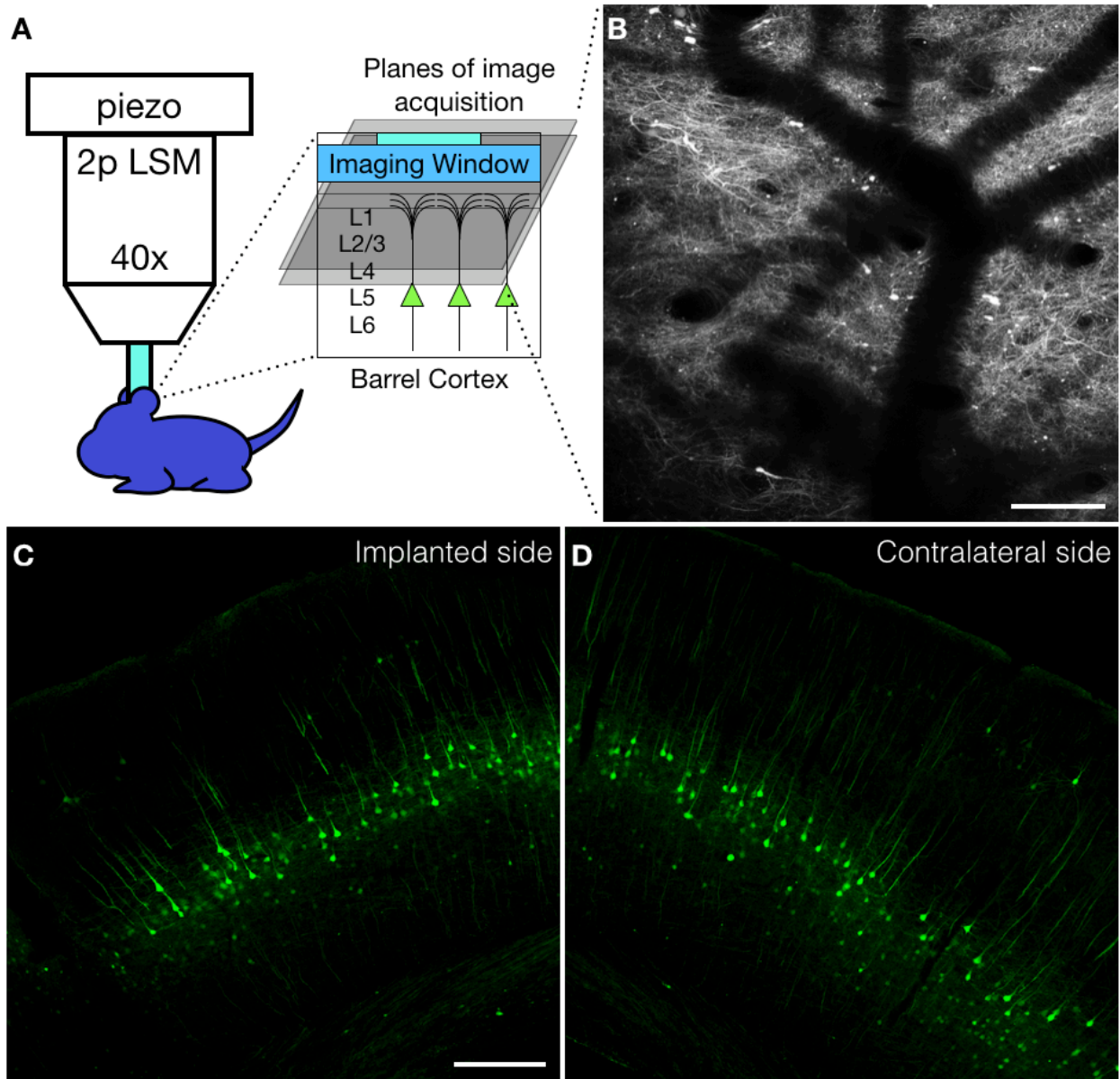
cortex was imaged to provide visual landmarks for subsequent acquisition of imaging fields (**Figure 2.2B**). Dendritic segments within  $\sim 100\ \mu\text{m}$  of the pial surface were imaged daily for five days (**Figure 2.2A**), followed by whisker trimming or sham treatment and five more days of imaging (**Figure 2.1**). When possible, the same dendritic segment was tracked throughout the entire 10-day experiment (**Figure 2.3D**). All analyzed segments were tracked at least for 5 days (baseline imaging or post-trim/sham treatment). Segments were  $15\text{--}45\ \mu\text{m}$  and contained 6-35 spines.



### Figure 2.1 - Experimental timeline

Thy1-YFP-H and Thy1-YFP-H x SRGAP2-KD mice were implanted with stainless steel headposts and glass imaging windows over their left barrel cortex at  $\sim\text{P150}$ . Animals were habituated to the imaging apparatus two weeks post-surgery for one week, followed by 5 days of daily baseline imaging. The mice were then subjected to either bilateral whisker trimming or a sham treatment (see Methods) immediately before the 6th day of imaging and every two days following, while imaging for 5 more days. 12 mice were imaged total, 3 each for Trim/Sham for both WT and SRGAP2 knockdown conditions.

Various other studies have generated data of dendritic spines through collecting a single static image/stack of a given field of view (e.g. **Grutzendler et al. 2002**). However, this is susceptible to motion artifacts when the animal moves, which could cause double counting of a given spine or missing one altogether.

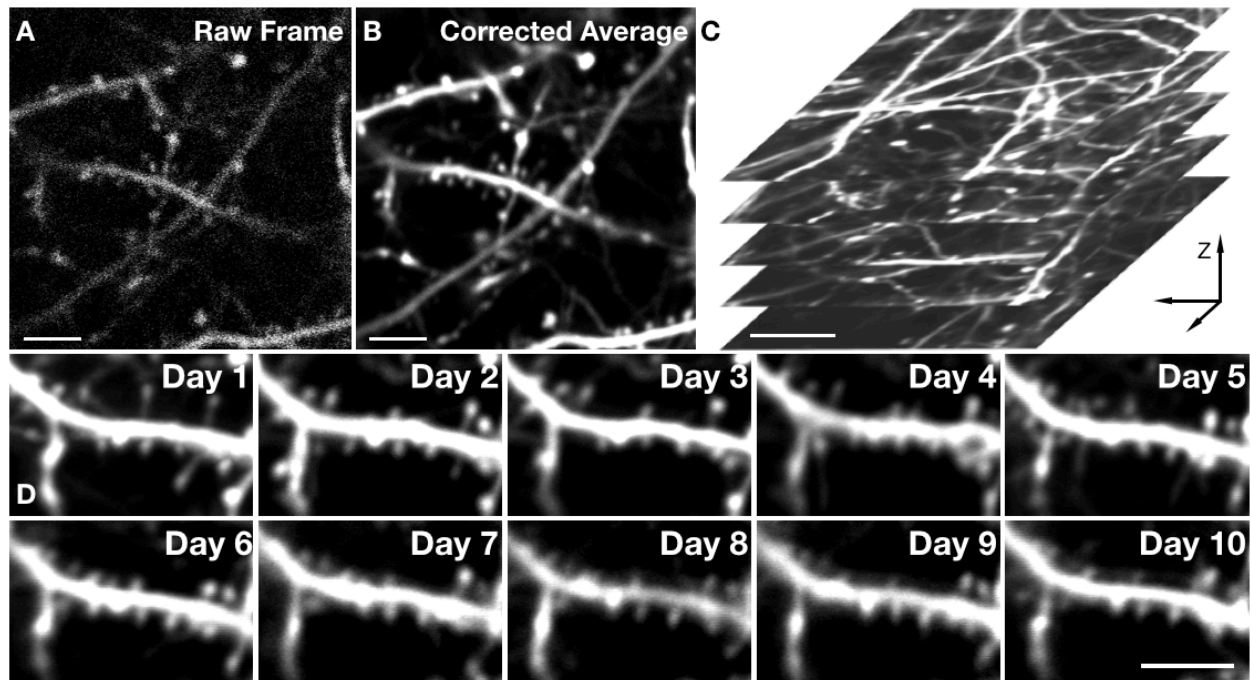


### Figure 2.2 - Imaging schematic

**A.** Schematic of the imaging apparatus. Z-stacks were acquired using a piezoelectric crystal in conjunction with a resonant scanner, allowing rapid image-acquisition (see Methods text for more details). **B.** Example of the imaging field of view in barrel cortex. The vasculature pattern allowed us to identify the desired dendritic segments repeatedly. Scale bar 100  $\mu\text{m}$ . **C-D.** Coronal confocal images of the implanted barrel cortex (**C**) and the contralateral barrel cortex (**D**) in Thy1-YFP mice. Layer 5 pyramidal neurons are labeled sparsely with YFP. Scale bar 250  $\mu\text{m}$ .

To alleviate the impact of motion artifacts, and to increase our signal-to-noise ratio, we collected 200 image-stacks of a given field-of-view using a resonance scanner in combination

with a piezo-electric device. We then used a motion-correction algorithm based on the Hidden-Markov Model (Kaifosh et al. 2013, Kaifosh et al. 2014) to correct for displacement in individual images in x, y, and z directions, which were then averaged to generate our final image for analysis (Figure 2.3A-C). Because our data was averaged from a large number of individual images, we minimized the chance of miscounting spines due to motion aberrations or low signal. Additionally, this may allow us to generate more usable data per animal, as we are able to more consistently monitor the same dendrites over several days and weeks.



### Figure 2.3 - Data acquisition and processing

Imaged layer 5 apical dendrite segments. **A.** Example of one frame of raw data acquired. **B.** Area from A post-motion-correction and averaging (see Methods text). Scale bar 5  $\mu\text{m}$ . **C.** Example acquired Z-stack, post-motion-correction. Scale bar 10  $\mu\text{m}$ . **D.** Example of an imaged dendritic segment over 10 days. Whisker trimming or sham treatment (see Methods text) occurred immediately before day 6 of imaging. Scale bar 5  $\mu\text{m}$ .

### Whisker trimming

Following five days of baseline imaging, whisker trimming was performed daily by cutting the mystacial vibrissae of both whisker-pads to skin level with a pair of scissors under a dissecting

microscope (**Figure 2.1**). For sham treatment, scissors were brushed across the whisker-pads without cutting, with conditions identical to the trim treatment. Trimming was performed every two days.

## **Histology**

Mice were perfused (transcardial PBS, 4% formaldehyde), and their brains were postfixed in 4% paraformaldehyde for 2 hours and sectioned (100  $\mu$ m). Confocal stacks were taken using a Olympus FluoView1000 microscope (**Figure 2.2C-D**).

## **Motion correction**

Acquired image stacks were corrected for motion in x, y, and z directions using a Hidden Markov Model as described previously (**Kaifosh et al. 2013, Kaifosh et al. 2014**), which is freely available in the SIMA package (**Kaifosh et al. 2014**).

## **Spine tracking**

A random subset (~10%) of spines identified as stable were confirmed to be within 0.7  $\mu$ m of their expected positions (see **Fu et al. 2012**). Spines were counted using ImageJ's multi-point tool.

## **Cluster analysis**

Image analysis was performed in ImageJ.

For newly formed spines, the distance to the nearest existing spine (spines that were present on the previous imaging day) was measured. In addition, existing spines within 5  $\mu$ m of dendrite

attachment of the spine were counted. Similarly, other newly formed spines (spines that were not present on the previous imaging day) within 5  $\mu\text{m}$  were counted. For these measurements, spines located  $<5 \mu\text{m}$  from image edges were ignored.

### **Normalizing turnover rates**

To determine whether the absolute number of spines undergoing turnover per day was different in SRGAP2 knockdown compared to WT (see Chapter 3), we calculated the ratio between the density multiplied by turnover rate of each condition:

$$\frac{\text{Density}_{\text{SRGAP2}} \times \text{Turnover}_{\text{SRGAP2}}}{\text{Density}_{\text{WT}} \times \text{Turnover}_{\text{WT}}}$$

Which yields the ratio between the absolute number of spines undergoing turnover per day in SRGAP2 knockdown compared to that of WT.

### **Statistical analysis**

All statistical tests are described within the results text or figure legends. All comparisons were two sided. Data reported in text as mean  $\pm$  SEM.

For survival fraction modeling, we fit our data to the exponential function:

$$\text{SF} = S_0 + (1 - S_0) \times e^{-t/\tau}$$

Where SF is the survival fraction,  $S_0$  is the proportion of permanent (non-dynamic) spines,  $t$  is the day, and  $\tau$  is the mean lifetime of dynamic spines.  $X^2$  value represents goodness of fit, with low values indicating small deviations between experimental and predicted values.

For Jenks Natural Break analysis, the goodness of variance fit (GVF) ranges from 0 to 1, with 1 representing perfect clustering.

## **Intracategory variance**

To reduce the chance that our results are due to data-skewing by single outlier animals, we compared the density and turnover results (see Chapter 3) within each category (WT baseline, WT sham, WT trim, MUT baseline, MUT sham, MUT trim; see Chapter 3 for details) using the Kruskal-Wallis test. For all measurements, the between-animals differences were not significant (Density -  $p_{WTbaseline} = 0.85$ ,  $p_{WTsham} = 0.73$ ,  $p_{WTtrim} = 0.56$ ,  $p_{MUTbaseline} = 0.93$ ,  $p_{MUTsham} = 0.75$ ,  $p_{MUTtrim} = 0.42$ ; Formation -  $p_{WTbaseline} = 0.44$ ,  $p_{WTsham} = 0.25$ ,  $p_{WTtrim} = 0.63$ ,  $p_{MUTbaseline} = 0.87$ ,  $p_{MUTsham} = 0.86$ ,  $p_{MUTtrim} = 0.75$ ; Elimination -  $p_{WTbaseline} = 0.85$ ,  $p_{WTsham} = 0.56$ ,  $p_{WTtrim} = 0.70$ ,  $p_{MUTbaseline} = 0.70$ ,  $p_{MUTsham} = 0.66$ ,  $p_{MUTtrim} = 0.87$ ).

## **List of metrics**

Spine density - number of spines per  $\mu\text{m}$  dendrite

Spine formation - number of newly formed spines / spines present on the previous day

Spine elimination - number of eliminated spines / spines present on the previous day

Survival fraction - number of spines on the current imaging day that were present on day one of imaging / total number of spines present on day one of imaging

## **CHAPTER 3**

Effects of SRGAP2 knockdown on spine dynamics in adult mice

## Summary

SRGAP2 localizes to dendritic spines and regulates synapse development. Expression of SRGAP2C, a human-specific paralog, phenocopies knockdown of SRGAP2, and appears to delay synaptic maturation and cause neoteny. The function of SRGAP2 in adults (P>150), however, remains unknown. Additionally, how SRGAP2 affects synaptic dynamics *in vivo* has yet to be determined. Here, we use chronic multi-day 2-photon scanning light microscopy (2PSLM) to image layer 5 apical dendritic spines in the barrel cortex of a Gene-trap mouse line where SRGAP2 expression is knocked down. We find that SRGAP2 knockdown increases spine density in adult mice while decreasing spine turnover rates. In addition, we find that SRGAP2 knockdown causes increased spine formation and a resultant increase in spine density in response to bilateral whisker trimming in adult mice, a treatment to which WT mice have no response. We also show that SRGAP2 knockdown changes the survival dynamics of dendritic spines by increasing the proportion of stable spines compared to the population of transient spines. In both WT and SRGAP2 knockdown conditions, whisker trimming does not affect the survival rate of existing spines. Finally, we show that the turnover rate of individual dendritic segments lies on a gradient, and the turnover rate does not correlate with the spine density of a segment. Our results show that SRGAP2 affects synaptic dynamics in adults, and changes how synaptic networks respond to environmental changes.

## Introduction

SRGAP2 has important roles in regulating synaptic dynamics. Previous findings suggest that the presence of SRGAP2C in humans serves to delay cortical maturation in humans, promoting neoteny (Charrier et al 2012, Fossati et al. 2016). Expression of SRGAP2C or the knockdown



of SRGAP2 in mice serve similar functions - delayed cortical migration, delayed synapse maturation (both excitatory and inhibitory), and eventually increased synapse density and size (**Charrier et al. 2012, Fossati et al. 2016**). Therefore, the level of SRGAP2 activity (partially controlled by SRGAP2C expression in humans) may regulate how phenotypically “human” cortical synaptic organization becomes (**Charrier et al. 2012, Fossati et al. 2016**).

Past studies on SRGAP2 have determined how the gene affects mice at adolescent and young adult ages (~P20-P70; **Charrier et al. 2012, Fossati et al. 2016**). However, other labs have shown that synaptic organizational changes, while most striking at young ages, continues well past P70 and through the age of several months to a year. In particular, in mouse barrel cortex, layer 5 pyramidal neurons exhibit a continued decrease in dendritic spine density well past 4 months of age (**Zuo et al. 2005**). As we hypothesize that SRGAP2 delays the maturation of synaptic organization, it is of great interest to determine how SRGAP2 manipulation affects the synapses of these truly “adult” animals.

As dendritic spines are a substrate for memory and learned experiences (see Chapter 1), how SRGAP2 manipulation affects their dynamics will provide valuable insight into how evolution changed the capacity of the human brain to respond to stimuli. Spine dynamics vary across different brain areas, between cell types, and even within the different compartments of a given cell (**Sala and Segal 2014**).

Here, we specifically investigate the apical dendrites of layer 5 pyramidal neurons in the mouse barrel cortex. This compartment is readily accessible to chronic 2PSLM imaging, and other studies have determined that spine density in this area decreases rapidly through early maturation due to a spine elimination rate that vastly outpaces spine formation (**Zuo et al. 2005**).

While the decrease slows throughout development, the effect still persists at adult ages (P>150, **Zuo et al. 2005**).

Importantly, in wild-type animals, whisker trimming does not noticeably alter spine dynamics at P>150 on time-scales shorter than 2 months, where the cumulative effects of slightly increased spine elimination can be observed (**Zuo et al. 2005**). In contrast, the increased spine elimination can be seen on an order of weeks in juvenile mice (**Zuo et al. 2005**). If SRGAP2 knockdown promotes neoteny by delaying cortical/spine maturation, as we hypothesize, we expect a reversion to younger phenotypes, where whisker trimming will affect spine dynamics on shorter time scales.

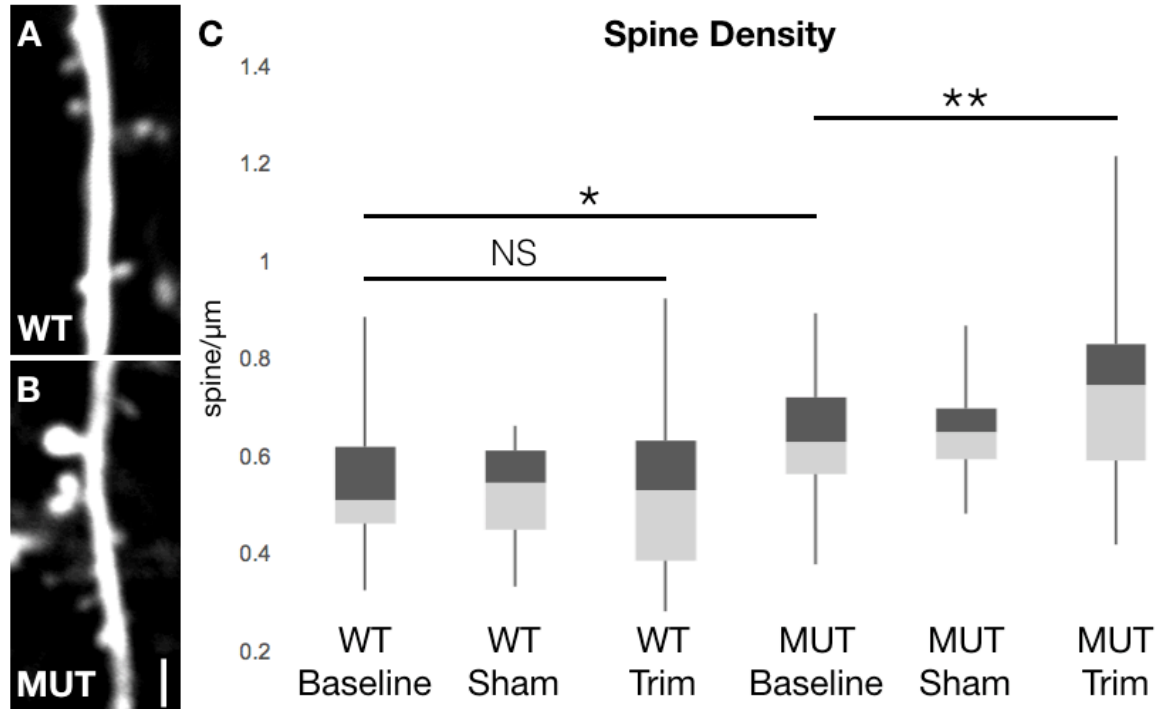
To investigate how SRGAP2 affects cortical synapses in adults, we imaged spines on the apical dendrites of layer 5 pyramidal neurons in the barrel cortex of both WT and SRGAP2 knockdown (using a Gene-trap mouse line; see Chapter 2 for details) adult mice (P>150, as defined by previous studies; **Zuo et al. 2005**). In addition to measuring how SRGAP2 knockdown affects spine density in adult mice, 2PSLM also allows us to determine how SRGAP2 knockdown alters spine dynamics for the first time, as we are able to perform longitudinal imaging of the same dendritic segments rather than simply fixed time as in earlier studies of SRGAP2 (e.g., **Charrier et al. 2012**, **Fossati et al. 2016**, **Guerrier et al. 2009**).

## **Results**

### ***SRGAP2 knockdown increases baseline spine density in adult mice***

While Charrier et al. showed that SRGAP2 knockdown increases synaptic density at both juvenile (P~20) and young adult (P~70) ages, it is unknown whether this effect persists later into adulthood (**Charrier et al. 2012**). To determine how SRGAP2 knockdown throughout life

affects synaptic density in older mice, we imaged apical dendritic spine segments in layer 5 pyramidal neurons in the barrel cortex of both WT and SRGAP2 KD adult mice (ages ranged from P154-P171 at the start of imaging) over five days, and measured spine densities for these segments (see Chapter 2 for details).

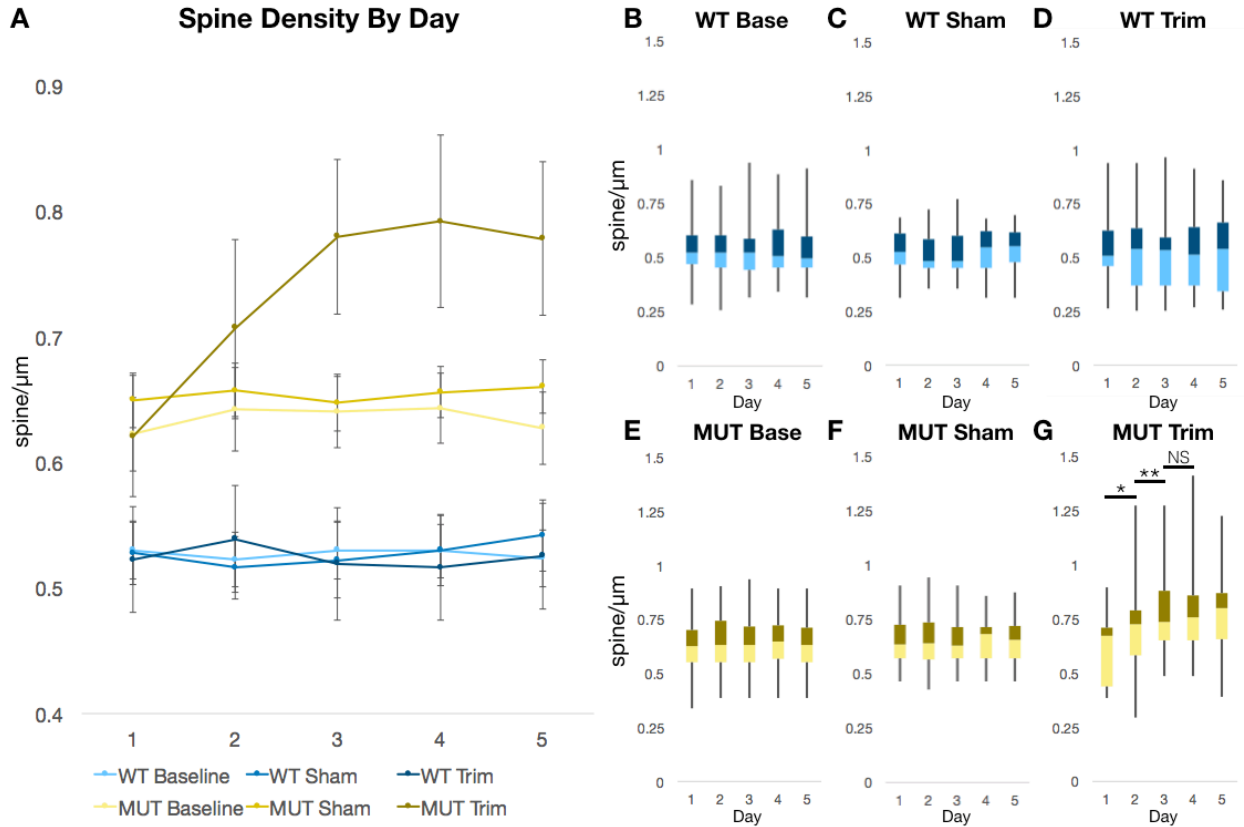


**Figure 3.1 - Spine density on layer 5 apical dendrites in barrel cortex in adult WT and SRGAP2 KD animals**

**A-B.** Example layer 5 apical dendritic segments expressing YFP (Thy1-YFP H line) in wild-type (WT, **A**) and SRGAP2 KD (MUT, **B**) mice at P<150. Scale bar 2 μm. **C.** Box plots showing the density of imaged layer 5 apical dendritic segments at baseline and following whisker trimming or sham treatment. Plot ranges - minimum, 25%, 50%, 75%, maximum. Sample sizes (dendritic segments):  $n_{WTbaseline} = 48$ ,  $n_{WTsham} = 23$ ,  $n_{WTtrim} = 26$ ,  $n_{MUTbaseline} = 42$ ,  $n_{MUTsham} = 20$ ,  $n_{MUTtrim} = 21$ . \* $p < 0.0002$ , \*\* $p < 0.05$ , NS  $p = 0.5$ , Kruskal-Wallis test followed by Mann-Whitney U test.

SRGAP2 KD mice show a marked increase in average spine density compared to WT animals (**Figure 3.1C**;  $0.53 \pm 0.02$  spines/μm in WT to  $0.63 \pm 0.02$  spines/μm in MUT,  $p < 0.0002$ , Kruskal-Wallis test followed by Mann-Whitney U test). This is consistent with

previous results showing a ~20-40% increase in density from WT animals to SRGAP2 knockdown animals in layer 5 pyramidal cell dendrites of somatosensory cortex of juvenile to young adult (P21-P75) mice (Charrier et al. 2012).



**Figure 3.2 - Layer 5 apical dendritic segment spine density over the course of 5 imaging days in adult WT and SRGAP2 KD mice**

**A.** Spine density of dendritic segments for WT and SRGAP2 KD (MUT) mice at baseline and following whisker trimming or sham treatment by day. Error bars represent SEM. **B-G.** Individual box plots showing spine density over the imaging period for each condition. Plot ranges - minimum, 25%, 50%, 75%, maximum. \* $p < 0.01$ , \*\* $p < 0.02$ , NS  $p = 0.17$ , Friedman test followed by Wilcoxon signed-rank test. Sample sizes (dendritic segments):  $n_{WTbaseline} = 48$ ,  $n_{WTsham} = 23$ ,  $n_{WTtrim} = 26$ ,  $n_{MUTbaseline} = 42$ ,  $n_{MUTsham} = 20$ ,  $n_{MUTtrim} = 21$ .

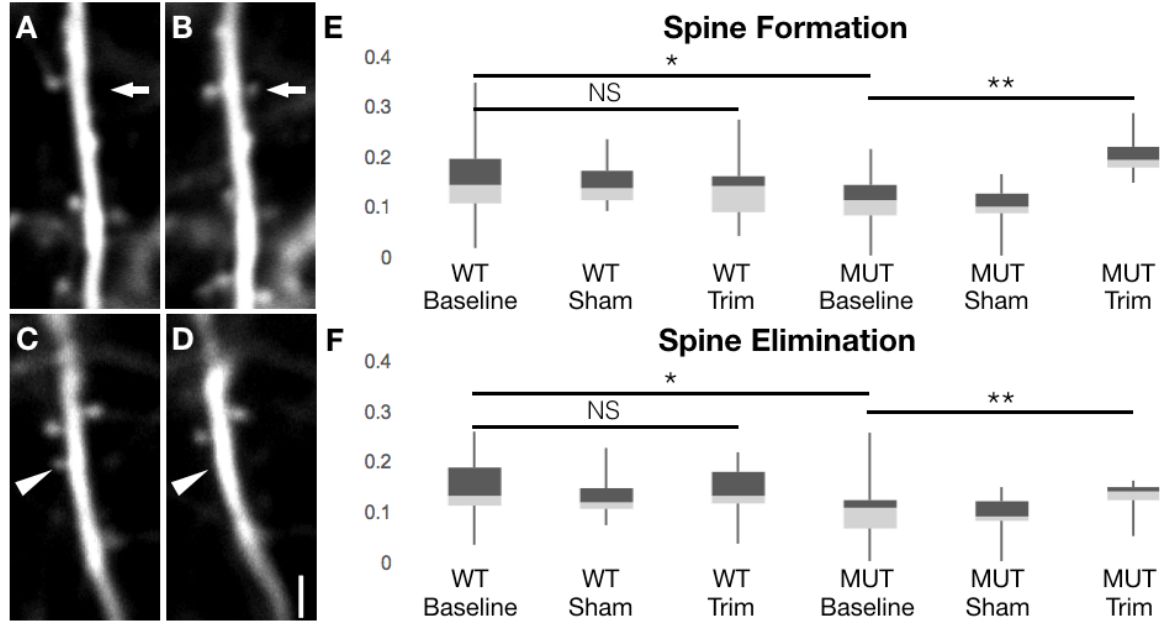
***SRGAP2 knockdown allows spine density to increase in response to bilateral whisker trimming in adult mice***

In juvenile animals, bilateral whisker trimming increases the density of layer 5 apical dendritic spines in the barrel cortex by decreasing their elimination rate. In WT animals, the ability of these spines to respond to bilateral whisker trimming is severely attenuated by P150 (Zuo et al. 2005). However, as SRGAP2 knockdown promotes neoteny in young adults (Charrier et al. 2012, Fossati et al. 2016), we expected that SRGAP2 knockdown may allow older mice to more efficiently modulate their synaptic architecture in response to whisker trimming. To determine how synaptic density is affected by whisker trimming in the barrel cortex of WT and SRGAP2 KD mice, we next continuously imaged the same apical dendrites of layer 5 cells in barrel cortex for five days following either bilateral whisker trimming or a sham treatment (see Chapter 2 for details). As in the previous section, we measured spine densities for these segments.

As expected, WT mice did not respond to whisker trimming (Figure 3.1C;  $0.53 \pm 0.02$  spines/ $\mu\text{m}$  at baseline to  $0.52 \pm 0.04$  spines/ $\mu\text{m}$  post-trimming,  $p=0.5$ , Kruskal-Wallis test followed by Mann-Whitney U test). However, SRGAP2 KD mice exhibited a further increase in spine density after whisker trimming (Figure 3.1C;  $0.63 \pm 0.02$  spines/ $\mu\text{m}$  at baseline to  $0.74 \pm 0.06$  spines/ $\mu\text{m}$  post-trim,  $p<0.05$ , Kruskal-Wallis test followed by Mann-Whitney U test).

Further breakdown of density measurements by day show that the increase in density occurs over the first two days following whisker trimming, after which the density appears to plateau over the remainder of the imaging period (Figure 3.2A,G; density progression:  $0.62 \pm 0.05$ ,  $0.71 \pm 0.07$ ,  $0.78 \pm 0.06$ ,  $0.79 \pm 0.07$ ,  $0.78 \pm 0.06$  spines/ $\mu\text{m}$ ;  $p<0.01$ , Friedman test followed by Wilcoxon signed rank tests). Comparisons of daily density for the other five conditions (WT baseline, WT sham, WT trim, MUT baseline, MUT sham) did not reveal any

significant differences (**Figure 3.2**; Friedman tests,  $p$  ranged from 0.35 to 0.92). These results suggest that SRGAP2 knockdown primes layer 5 apical dendrites of barrel cortex to respond to whisker trimming in adult mice.



**Figure 3.3 - Spine turnover on layer 5 apical dendrites in barrel cortex in adult WT and SRGAP2 KD animals**

**A-D.** Example layer 5 apical dendritic segments exhibiting formed (**A, B**) and eliminated (**C, D**) dendritic spines. Arrow marks a formed spine; arrowhead marks an eliminated spine. Scale bar 2  $\mu\text{m}$ . **E-F.** Box plots showing formation (**E**) and elimination (**F**) rates of dendritic spines of imaged layer 5 apical dendritic segments at baseline and following whisker trimming or sham treatment. Plot ranges - minimum, 25%, 50%, 75%, maximum. **E.** \* $p < 0.01$ , \*\* $p < 0.00001$ , NS  $p < 0.398$ ; **F.** \* $p < 0.002$ , \*\* $p < 0.01$ , NS  $p = 0.329$ , Kruskal-Wallis test followed by Mann-Whitney U test. Sample sizes (dendritic segments):  $n_{WT\text{baseline}} = 48$ ,  $n_{WT\text{sham}} = 23$ ,  $n_{WT\text{trim}} = 26$ ,  $n_{MUT\text{baseline}} = 42$ ,  $n_{MUT\text{sham}} = 20$ ,  $n_{MUT\text{trim}} = 21$ .

### ***SRGAP2 knockdown decreases the baseline rate of synaptic turnover in adult mice***

No previous studies of SRGAP2 have explored how synaptic dynamics are affected with SRGAP2 manipulation. Determining how synaptic turnover is affected by SRGAP2 knockdown is critical for elucidating the mechanisms of how SRGAP2 regulates the plasticity of excitatory synapses, as well as exploring the functional consequences of SRGAP2 loss. As we imaged

dendritic spines *in vivo* over a period of up to ten days (see Chapter 2 for details), we were able to measure how SRGAP2 knockdown affects synaptic turnover and compare this to WT mice.

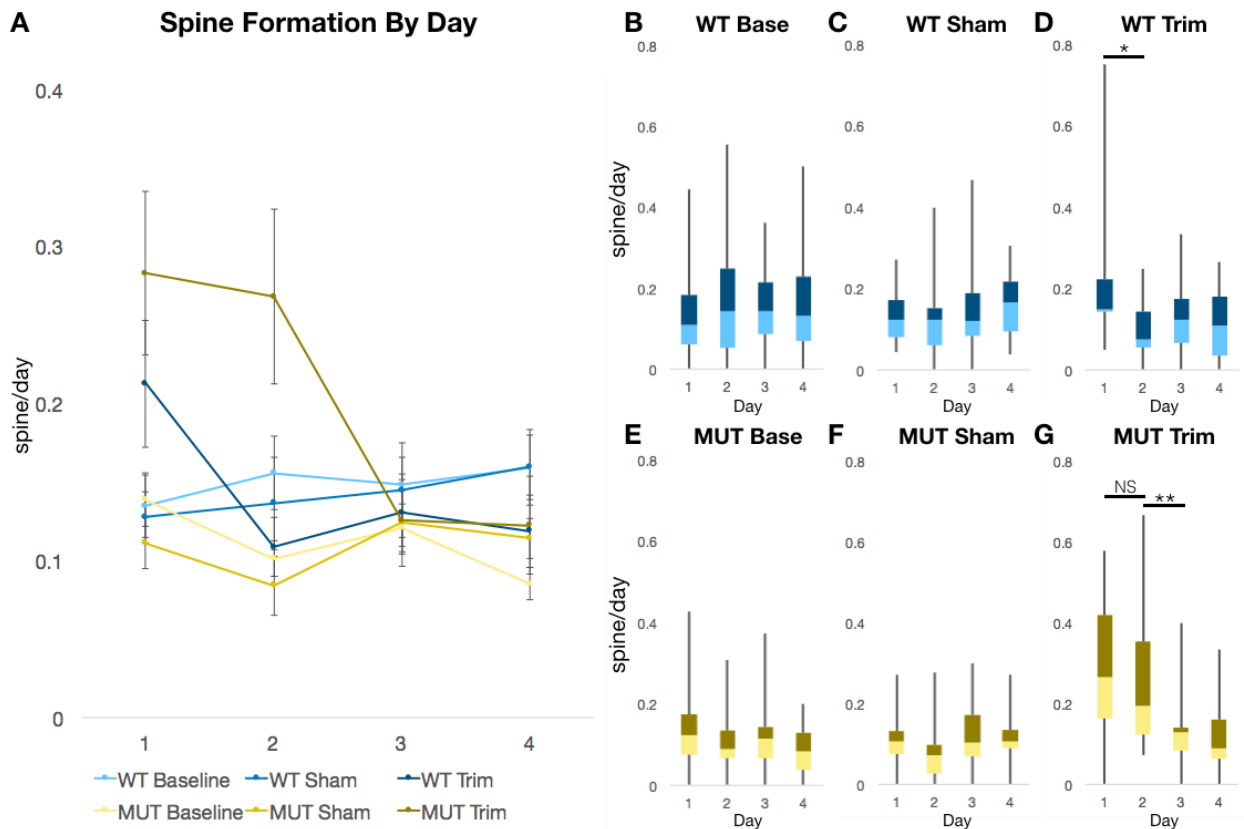
We find that SRGAP2 knockdown decreases the rate of spine turnover compared to WT animals (**Figure 3.3E,F**;  $p < 0.02$ , Kruskal-Wallis test followed by Mann-Whitney U test). In WT animals, the rate of formation and elimination are  $0.150 \pm 0.013$  and  $0.144 \pm 0.010$  spines/day, respectively, while in SRGAP2 knockdown animals the same rates are  $0.112 \pm 0.009$  and  $0.104 \pm 0.009$  spines/day (**Figure 3.3E,F**).

While the relative rate of turnover is lower in SRGAP2 knockdown animals compared to WT, it is possible that the absolute number of spines turned over per day is similar due to the increased density seen with SRGAP2 knockdown. To account for this, we normalized the rates to control for density (see Chapter 2 for details). We find that SRGAP2 knockdown animals exhibit 9.9% less spine turnover than WT animals, suggesting that SRGAP2 knockdown decrease the absolute number of plastic spines.

### ***SRGAP2 knockdown increases spine formation in response to bilateral whisker trimming in adult mice***

While the ability of SRGAP2 knockdown adult mice to respond to whisker trimming appears to be a form of neoteny, it is possible that the response differs mechanistically from that of juvenile mice, despite similar effects. In juvenile mice, whisker trimming lowers the elimination rate of spines while not notably affecting formation rates, causing increased spine density (**Zuo et al. 2005**). Alternatively, increased formation without a commensurate increase in elimination can cause a similar outcome. To distinguish between these mechanistic possibilities, we examined how spine turnover is affected by whisker trimming in WT and SRGAP2 knockdown adult mice.

Consistent with previous studies (Zuo et al. 2005), we find no changes in overall turnover rates in WT mice in response to whisker trimming when we average daily turnover rates across the entire 5-day imaging session (Figure 3.3E,F;  $p=0.398$ , Kruskal-Wallis test followed by Mann-Whitney U test). Surprisingly, we observe a significant increase in the spine formation rate of SRGAP2 KD mice from  $0.112 \pm 0.009$  to  $0.200 \pm 0.011$  spines/day (Figure 3.3E;  $p<0.00001$ , Kruskal-Wallis test followed by Mann-Whitney U test). We also found a slight increase in the elimination rate in SRGAP2 KD animals, but to a much smaller degree than the increase in formation (Figure 3.3F;  $0.10 \pm 0.1$  to  $0.13 \pm 0.1$  spines/day,  $p<0.01$ , Kruskal-Wallis test followed by Mann-Whitney U test).

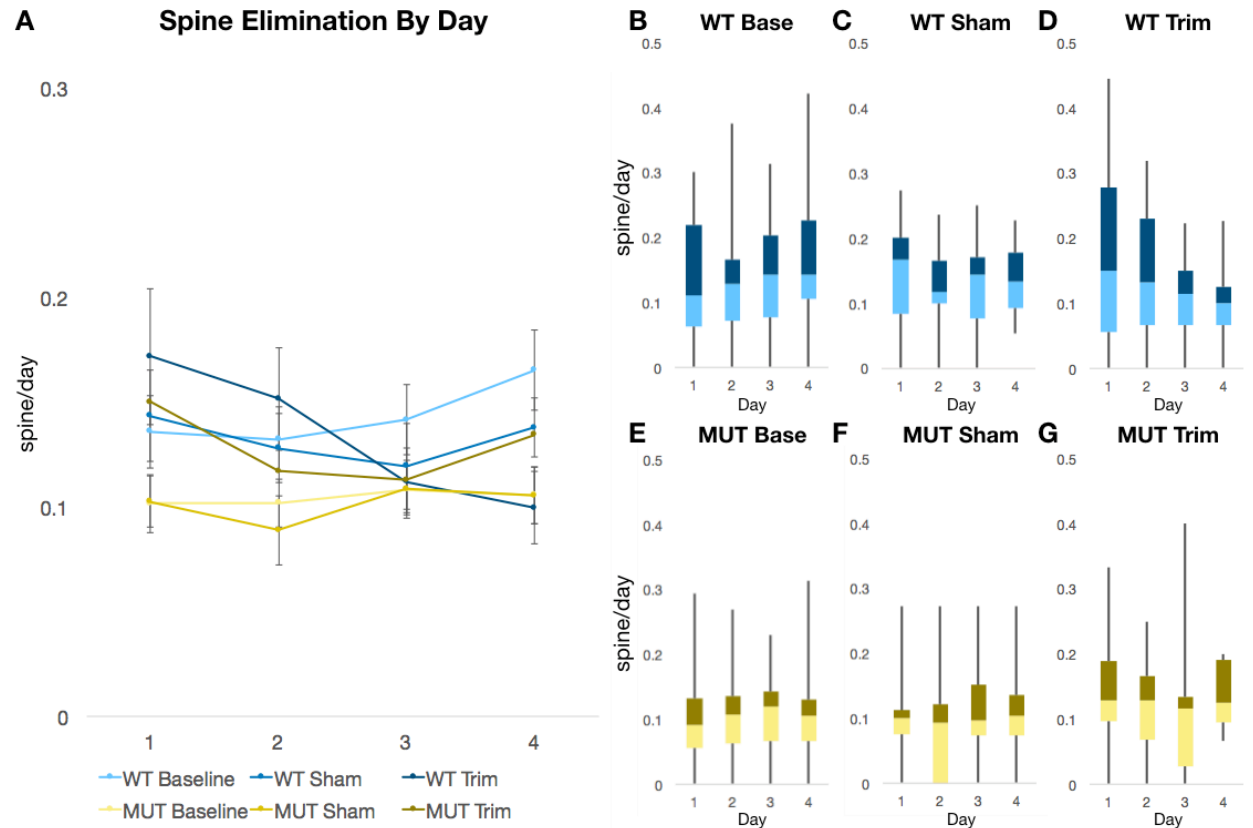


**Figure 3.4 - Layer 5 apical dendritic segment spine formation over the course of 5 imaging days in adult WT and SRGAP2 KD mice**

**A.** Spine formation rate of dendritic segments for WT and SRGAP2 KD (MUT) mice at baseline and following whisker trimming or sham treatment by day. Error bars represent SEM. **B-G.** Individual box plots showing spine formation rate over the imaging period for each condition.



Plot ranges - minimum, 25%, 50%, 75%, maximum. \* $p < 0.003$  \*\* $p < 0.006$ , NS  $p = 0.253$ , Friedman test followed by Wilcoxon signed-rank test. Sample sizes (dendritic segments):  $n_{WTbaseline} = 48$ ,  $n_{WTsham} = 23$ ,  $n_{WTtrim} = 26$ ,  $n_{MUTbaseline} = 42$ ,  $n_{MUTsham} = 20$ ,  $n_{MUTtrim} = 21$ .



**Figure 3.5 - Layer 5 apical dendritic segment spine elimination over the course of 5 imaging days in adult WT and SRGAP2 KD mice**

**A.** Spine elimination rate of dendritic segments for WT and SRGAP2 KD (MUT) mice at baseline and following whisker trimming or sham treatment by day. Error bars represent SEM. **B-G.** Individual box plots showing spine elimination rate over the imaging period for each condition. Plot ranges - minimum, 25%, 50%, 75%, maximum. Sample sizes (dendritic segments):  $n_{WTbaseline} = 48$ ,  $n_{WTsham} = 23$ ,  $n_{WTtrim} = 26$ ,  $n_{MUTbaseline} = 42$ ,  $n_{MUTsham} = 20$ ,  $n_{MUTtrim} = 21$ .

Examining the turnover rates by day reveals that the increased formation rate persists during the first two days following whisker trimming, after which it returns to baseline (**Figure 3.4G**; Day 1-2 -  $0.28 \pm 0.05$  spines/day, Day 2-3 -  $0.27 \pm 0.06$  spines/day, Day 3-4 -  $0.13 \pm 0.03$  spines/day, Day 4-5 -  $0.12 \pm 0.03$  spines/day;  $p < 0.05$  Friedman test followed by Wilcoxon

signed-rank test). The increase over the first two days after whisker trimming were similar in magnitude ( $p=0.253$ , Wilcoxon signed-rank test; **Figure 3.4G**). Elimination rate does not significantly vary by day in any of the six conditions (**Figure 3.5**;  $p>0.35$  for all conditions, Friedman test). Therefore, the increase in density we observe above can be attributed to a dramatic increase in formation rate.

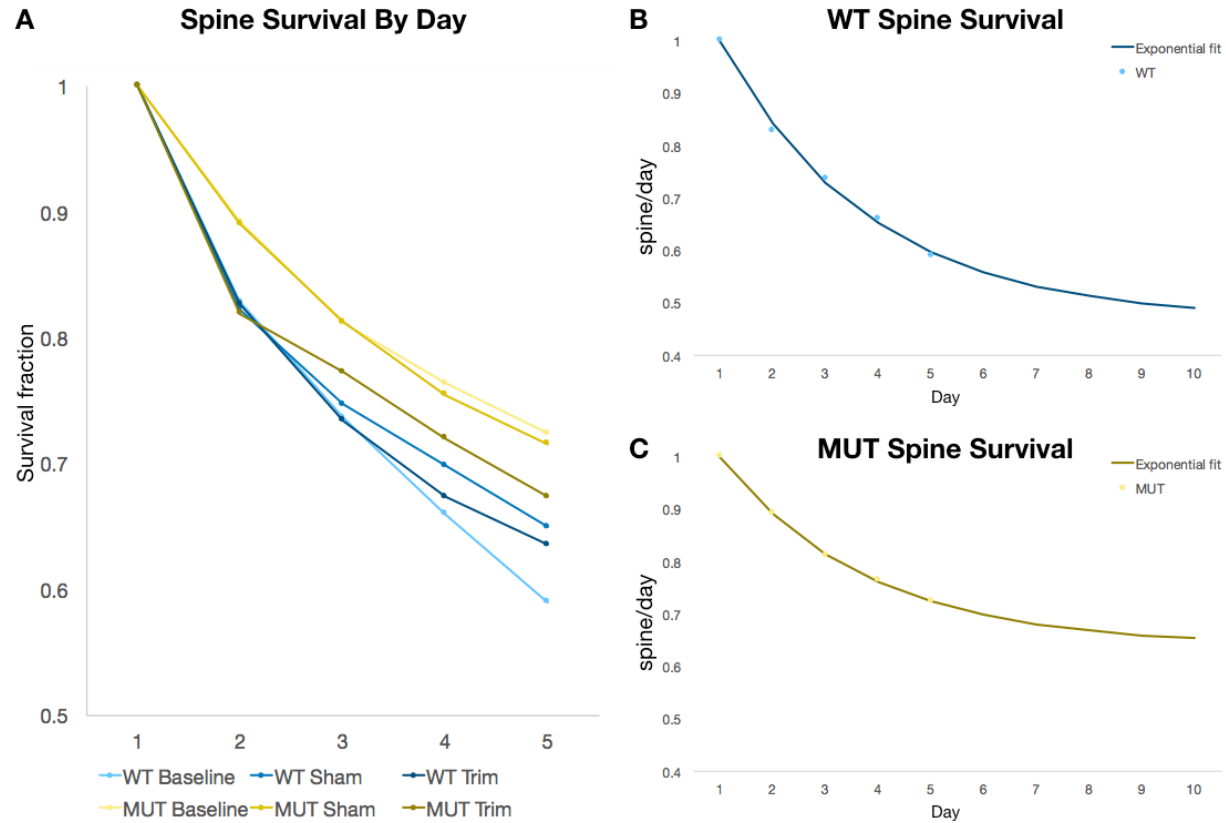
Unexpectedly, when breaking down turnover by day, we also note an increase in formation rate on the first day following whisker trimming in WT mice (**Figure 3.4D**; Day 1-2 -  $0.21 \pm 0.04$  spines/day, Day 2-3 -  $0.11 \pm 0.02$  spines/day, Day 3-4 -  $0.13 \pm 0.02$  spines/day, Day 4-5 -  $0.12 \pm 0.02$  spines/day;  $p<0.05$  Friedman test followed by Wilcoxon signed-rank test). However, this effect did not manifest in significant changes in spine density from day to day (**Figure 3.2D**;  $p=0.36$ , Friedman test).

### ***SRGAP2 knockdown increases the lifetime of dendritic spines***

To determine how SRGAP2 knockdown affects spine survival, we next analyzed survival curves (see Chapter 2 for details) for each of six conditions (**Figure 3.6A**; WT baseline, WT sham, WT trim, MUT baseline, MUT sham, MUT trim) by tracking all spines that were present on the first day of imaging and observing if/when they were eliminated over the course of five days of imaging. We found a significant difference between the survival curves of WT baseline and MUT baseline conditions (**Figure 3.6B,C**;  $p<0.00002$ , Cochran-Mantel-Haenszel test followed by Log-rank test).

To compare the WT baseline and MUT baseline curves, we fit our data to exponential decay functions (see Chapter 2 for details). Through this analysis, we can distinguish between

populations of transient spines and longer lasting persistent spines. In addition, we can determine the mean lifetimes of transient spines.



**Figure 3.6 - Survival of layer 5 apical dendritic spines over the course of 5 imaging days in adult WT and SRGAP2 KD mice**

**A.** Survival fractions of WT and SRGAP2 KD (MUT) mice at baseline and following whisker trimming or sham treatment by day. **B-C.** Survival fraction of spines at baseline in WT and MUT animals with plotted exponential fit (see text and methods for details). The horizontal asymptote (proportion of stable spines) in WT and MUT conditions are 0.47 and 0.64 respectively, while the mean lifetime of spines is 2.8 days for both conditions. Sample sizes (spines):  $n_{WTbaseline} = 539$ ,  $n_{WTsham} = 252$ ,  $n_{WTtrim} = 291$ ,  $n_{MUTbaseline} = 528$ ,  $n_{MUTsham} = 344$ ,  $n_{MUTtrim} = 176$ .

In WT animals, transient spines have a mean lifetime of 2.8 days, while 47% of spines are long-lasting ( $X^2 = 0.0003$ ). In SRGAP2 knockdown animals, transient spines have a mean lifetime of 2.8 days, while 64% of spines are long-lasting ( $X^2 = 0.00002$ ). This is consistent with

the idea that SRGAP2 knockdown decreases the proportion of plastic spines, as suggested by the decreased turnover rates it induces (**Figure 3.3**). Interesting, the similar mean lifetimes of the transient populations in both conditions suggests that the dynamics of the transient spines remain comparable to WT when SRGAP2 is knocked down.

#### ***Whisker trimming does not affect the survival of existing spines***

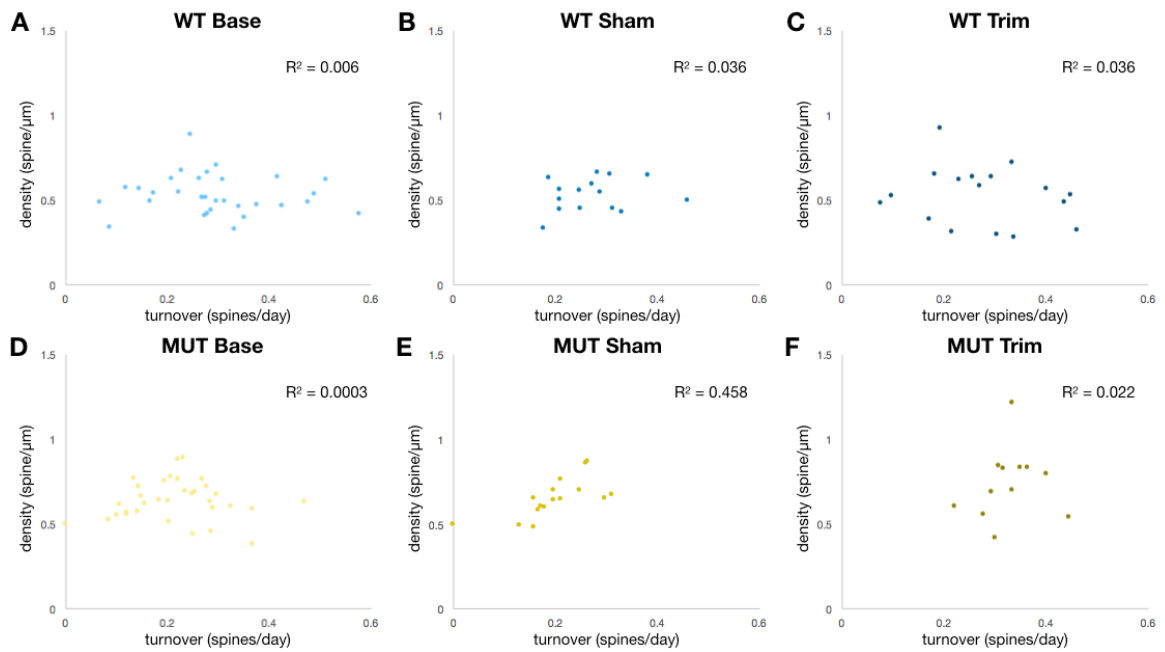
To determine whether whisker trimming affects survival of existing spines in both WT and SRGAP2 knockdown conditions, we compared the survival curves of dendritic spines before and after whisker trimming. In both WT and SRGAP2 knockdown animals, we find that whisker trimming does not significantly affect spine survival (**Figure 3.6A**;  $p=0.22$  for WT base vs WT trim,  $p=0.17$  for MUT base vs MUT trim, Cochran-Mantel-Haenszel test followed by Log-rank tests).

#### ***Dendritic segments have a gradient of turnover rates***

As there are populations of stable spines and transient spines, it is possible that certain dendritic segments are plastic while others have essentially stable spine populations. To attempt to determine whether there are indeed two populations of dendritic segments, we performed Jenks Natural Breaks analysis on our dendritic segments in each of six conditions (WT base, WT sham, WT trim, MUT base, MUT sham, MUT trim). In each case, the turnover rates fail to stratify into distinct clusters (turnover rate = formation rate + elimination rate; GVF = 0.62, 0.63, 0.69, 0.62, 0.54, and 0.61 for each condition respectively).

### ***The density of a dendritic segment does not correlate with its turnover rate***

It is possible that the density of a given dendritic segment affects its relative turnover rate. To see if this is true for WT animals, and to determine whether SRGAP2 knockdown affects this relationship, we performed linear regressions on our data (where turnover rate = formation rate + elimination rate as in above; **Figure 3.7**). For each of six conditions (WT base, WT sham, WT trim, MUT base, MUT sham, MUT trim), the  $R^2$  values were 0.006, 0.036, 0.036, 0.0003, 0.458, 0.022 respectively, indicating that there is no relationship between the density of a dendritic segment and its turnover rate. The p values for the slopes of each condition were 0.69, 0.50, 0.47, 0.92, 0.004, and 0.64 respectively. While the MUT sham condition showed significance, none of our analysis revealed differences between MUT baseline and MUT sham conditions; therefore, while this warrants further study, it may represent a statistical anomaly.



**Figure 3.7 - Spine density vs turnover for layer 5 apical dendrites in barrel cortex in adult WT and SRGAP2 KD mice**

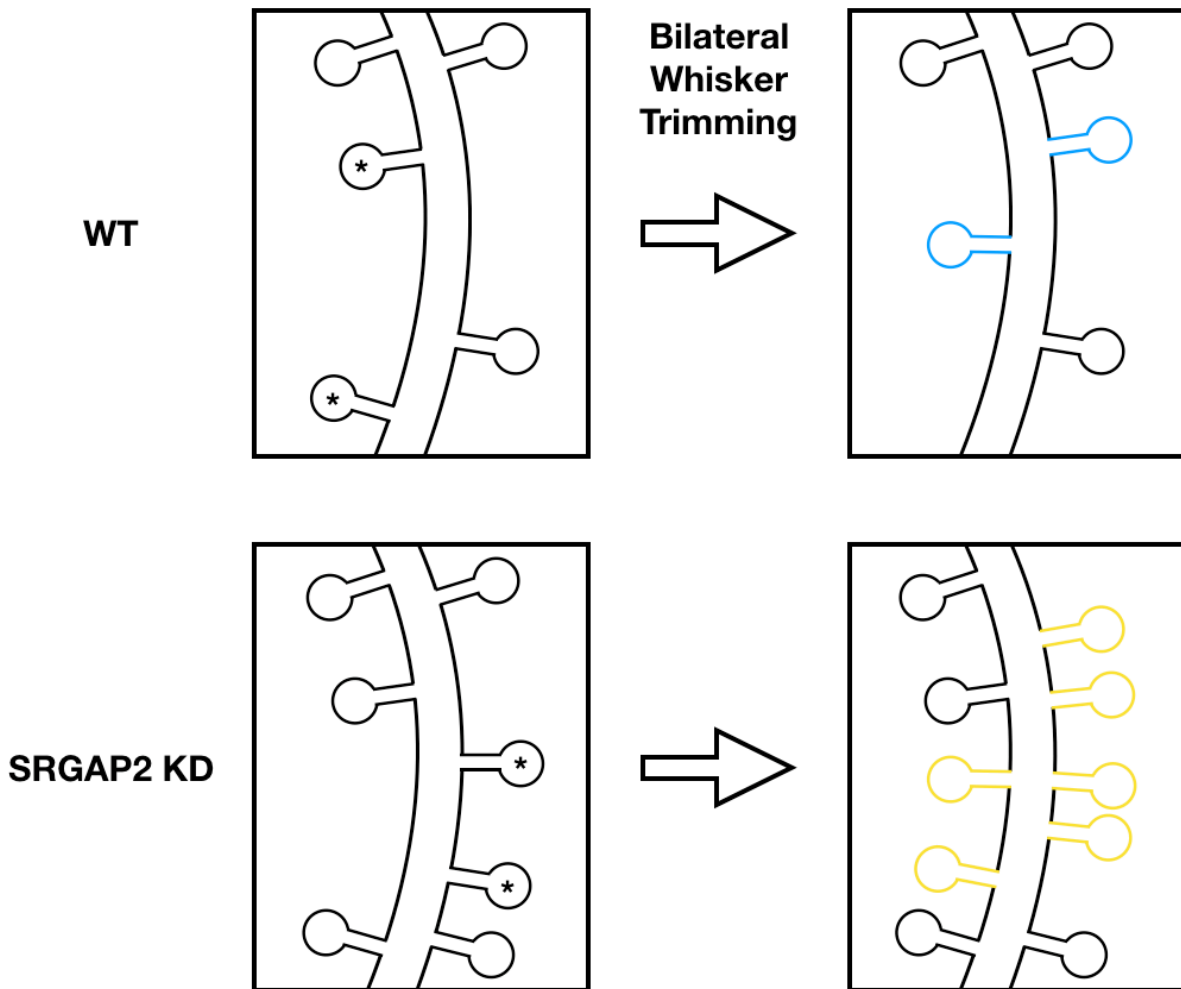
$p_{WTbaseline} = 0.69$ ,  $p_{WTsham} = 0.50$ ,  $p_{WTtrim} = 0.47$ ,  $p_{MUTbaseline} = 0.92$ ,  $p_{MUTsham} = 0.004$ ,  $p_{MUTtrim} = 0.64$ . Sample sizes (dendritic segments):  $n_{WTbaseline} = 48$ ,  $n_{WTsham} = 23$ ,  $n_{WTtrim} = 26$ ,  $n_{MUTbaseline} = 42$ ,  $n_{MUTsham} = 20$ ,  $n_{MUTtrim} = 21$

## Discussion

Here, we report the first detailed quantitative analysis of changes in spine dynamics resulting from SRGAP2 knockdown in adult ( $P > 150$ ) mice. In addition, we longitudinally monitor *in vivo* and analyze for the first time how SRGAP2 knockdown affects spine dynamics, as previous fixed time-point studies could not observe longitudinal effects (**Charrier et al. 2012, Fossati et al. 2016**). We find that SRGAP2 knockdown increases the baseline density of dendritic spines in layer 5 apical dendrites of barrel cortex in mice at  $P < 150$ . In addition, SRGAP2 knockdown increases the rate of spine formation in response to sensory deprivation *via* bilateral whisker trimming. (**Figure 3.8**).

When we measured spine turnover following bilateral whisker trimming, we observed a transient increase in spine formation for 1 day post-trimming in WT mice. To our knowledge, the increase in formation rate in response to whisker trimming has not been observed in the past. This could be due to our high imaging frequency (daily acquisition), as previous studies would not have observed this brief spike in formation without imaging every day following whisker trimming.

Interestingly, previous studies attribute the increased density seen through bilateral whisker trimming to a decrease in elimination rate, rather than an increased formation rate (**Zuo et al. 2005**). In the WT condition here, we see no noticeable increase in density or decrease in elimination rate with whisker trimming (**Figures 3.1C, 3.2D, 3.3F, 3.5D**). Similarly, we see no difference in the survival rate of existing spines (**Figure 3.6**). However, it remains possible that there is a small decrease in elimination that we are unable to detect, which may be compatible with the 2 month time course shown previously (**Zuo et al. 2005**). In this case, we may yet see the increase in density caused by a decrease in elimination rate.



**Figure 3.8 - SRGAP2 knockdown's effect on spine dynamics in layer 5 apical dendrites of adult barrel cortex**

SRGAP2 knockdown increases baseline spine density compared to wild-type and increases the rate of spine formation in response to bilateral whisker trimming, leading to an increase in overall spine density.

In contrast, the early increase in spine formation we observe during SRGAP2 knockdown does lead to an immediate increase in density following whisker trimming, and this increase in density persists throughout our imaging course (**Figures 3.1C, 3.2G, 3.3E, 3.4G**). In this case, the increased density cannot be attributed to a decreased elimination rate; indeed, we observe a

slight increase in elimination rate, possibly as a homeostatic response to compensate for the increased formation (**Figure 3.3F**).

Compared to the increased spine formation seen in WT animals after whisker trimming, the early increase in spine formation seen with SRGAP2 knockdown is larger in both duration and amplitude (**Figure 3.4G**). This can be interpreted as SRGAP2 knockdown increasing the ability of the circuit to respond to environmental changes by enhancing the potential for increased spine formation, possibly encouraging new network connections. Future work should address the behavioral consequences of these circuit changes. For example, we expect more efficient learning of whisker-related tasks when SRGAP2 is knocked down (e.g., see **Kuhlman et al. 2014**, see Chapter 5 for more discussion).

While the significant increase in spine density we find following whisker trimming in the SRGAP2 knockdown condition persists throughout the course of our experiment (**Figure 3.2G**), it is unclear how long this increase extends. In adolescent mice, allowing whiskers to grow back following prolonged sensory deprivation *via* trimming accelerates spine elimination, eventually bringing spine density down to control levels (**Zuo et al. 2005**). However, as whisker trimming in WT animals significantly affects spine elimination (**Zuo et al. 2005**), rather than spine formation in the case of SRGAP2 knockdown (**Figures 3.3E, 3.4G**), it is unclear whether removal of sensory deprivation in the case of SRGAP2 knockdown will cause decreased spine formation and/or increased spine elimination (both of which would bring spine density back down to base levels), or whether we may simply observe a more permanent increase in spine density. Longitudinal imaging extended past whisker trimming will be necessary to distinguish between these possibilities.



Through exponential fitting, we found that ~47% of spines are stable in WT animals, while ~63% are stable in SRGAP2 knockdown mice (**Figure 3.6**). These numbers are well in line with previous studies, which reported that between ~35% to >60% of spines in layer 5 apical dendrites are a stable population, depending on imaging conditions (**Holtmaat et al. 2005, Trachtenberg et al. 2002, Xu et al. 2009**). Similarly, the mean lifetimes of ~2.8 days we see in our transient spine population matches well with the 1.5 - 5 day mean lifetimes reported by others (**Attardo et al. 2015, Holtmaat et al. 2005, Yang et al. 2009**).

With our data, we find that dendritic segment turnover rates exist on a gradient and cannot be easily clustered into “plastic” and “non-plastic” segments. This is true both at baseline and during bilateral whisker trimming. In other studies, targeted remodeling of specific dendritic segments have been reported under certain conditions, such as motor tasks specifically training forelimbs (**Wang et al. 2010; see also Yang et al. 2014**). However, bilateral whisker trimming targets the entire barrel cortex, and is consistent with our results. Future work could measure *in vivo* spine dynamics in response to chessboard trimming (trimming every other whisker) to determine whether dendrite-specific remodeling occurs, and how SRGAP2 affects these dynamics.

The logic of synaptic dynamics is not consistent across the mammalian cortex. In the binocular region of visual cortex, as in barrel cortex, sensory deprivation *via* temporary monocular deprivation leads to higher spine densities in the apical dendrites of layer 5 pyramidal neurons (**Hofer et al. 2009**). However, unlike what was reported previously in barrel cortex (**Zuo et al. 2005**), increased spine density in the visual cortex results from increased spine formation, rather than decreased spine elimination (**Hofer et al. 2009**). Furthermore, removal of sensory deprivation through restoring binocular vision returns spine formation rates to baseline levels,

but spine density remains elevated, with many spines that were formed during deprivation persisting (**Hofer et al. 2009**). In motor cortex, motor learning is accompanied by increased spinogenesis; however, this is followed by increased spine elimination, leading to a similar overall spine density to control mice (**Xu et al. 2009**).

Synaptic properties can also vary even within a cortical area, or within a single cell. For example, pyramidal neurons in hippocampal CA1 reportedly have a single transient population of dendritic spines, with no apparent population of stable ones (**Attardo et al. 2015**). Within the neocortex, the density and dynamics of layer 2/3 pyramidal neurons is reported to be significantly higher than layer 5 pyramidal cells at both 1 and 4 months and motor and barrel cortex (**Tjia et al. 2017**). Additionally, apical tufts of layer 4 pyramidal neurons of barrel cortex exhibit increased spine loss following whisker trimming, compared to the increased spine density seen in the apical tufts of layer 5 pyramidal cells (**Miquelajauregui et al. 2015**). How SRGAP2 affects different dendritic compartments, cell types, and neocortical areas may also vary. For example, in hippocampal CA1 pyramidal neurons, conditional removal of SRGAP2 increases the spine density of basal and apical oblique dendrites by ~30%, but has no apparently effect on the density of apical tuft dendrites (**Polleux lab, unpublished observations**).

It is critical for future work to determine if effect of SRGAP2 on spine dynamics remains consistent throughout the brain, or if its functions vary in different cell populations. Indeed, it remains possible that SRGAP2 has specialized functions in heretofore unexplored brain regions. This notion is supported by the fact that the temporal pattern of SRGAP2 expression is nonuniform throughout the CNS; in the hippocampus, for example, the expression of SRGAP2 in CA1 and CA3 reverses early in development, with strong early expression in CA3 and weak expression in CA1 at P1 changing to weak CA3 expression and strong CA1 expression by P7

(**Bacon et al. 2009**). Interestingly, the rate of spine development can vary between cortical areas in the human brain (**Huttenlocher and Dabholkar 1997**); future research should determine whether variance in SRGAP2A and SRGAP2C expression is related to these differences.

In our data analysis, we treat all dendritic protrusions as dendritic spines, rather than categorize them into discrete spine and filopodia categories, like others (**Fu and Zuo 2011**). This is due to our somewhat varying image quality from day to day, which renders it difficult to unequivocally ascribe morphological characteristics to our putative spines with *in vivo* 2PSLM. As different dynamics have been reported for filopodia and full-fledged dendritic spines, it is possible that we lack a dimension in our analysis which can further elucidate how SRGAP2 affects spine dynamics. For example, it is possible that SRGAP2 decreases formation and elimination by reducing the proportion of dendritic protrusions that are filopodia, which are more dynamic than spines (**Fu and Zuo 2011**). Alternatively, SRGAP2 may not reduce the population of filopodia, but rather change the dynamic properties of filopodia themselves. Future work with more reliable high-resolution *in vivo* imaging is necessary to distinguish between such possibilities.

Importantly, while a typical distinction between filopodia and spines is that filopodia do not necessarily have functional synapses (**Matsuzaki et al. 2001**), recent work has shown that structures often classified as full dendritic spines may also not have functional synapses (e.g. **Villa et al. 2016**). Additionally, other results suggesting that dendritic protrusions exist on a continuous gradient may render such analysis less meaningful (**Tønnesen et al. 2014**). While dendritic spines are generally accepted as a proxy for excitatory synapses, future work should confirm that our observations hold when excitatory spines dynamics are more directly measured (see Chapter 5 for further discussion).

The absolute numbers on spine density and turnover obtained in *in vivo* studies vary significantly (e.g., **Hayashi-Takagi et al. 2014**, **Holtmaat et al. 2005**, **Tjia et al. 2017**, **Trachtenberg et al. 2002**, **Zuo et al. 2005**). While the cause of these differences is a subject of debate, one source of variation is likely the use of a thinned-skull preparation compared to open-skull windows, like the ones used for this dissertation. The inflammatory response and cellular remodeling involved in open-skull windows may increase observed spine turnover rates and affect density measurements (**Yang et al. 2010**). However, to perform repeated imaging of a cortical area with short intervals, an open-skull preparation is necessary. While this makes comparison of absolute numbers between studies difficult, the relative changes we observe remain instructive. Importantly, to our knowledge, there has been no reports of behavioral changes due to an open-window preparation, suggesting that trends observed using this method are credible.

## **CHAPTER 4**

SRGAP2's effect on the clustered formation of dendritic spines

## Summary

The formation of dendritic spines is not random on a dendritic segment, and multiple factors influence clustering in synaptic plasticity, which can influence the ability of cells to non-linearly integrate and process inputs. As SRGAP2 is a regulator of both inhibitory and excitatory synapses, it may play a role in regulating the distribution of synapses across dendritic arbors; indeed, it is known to alter the distribution of inhibitory synapses between dendritic spines and shafts. However, its role in clustered synaptic formation is unknown. Here, by longitudinally imaging the same dendritic segments over several days, we aim to monitor the dynamics of dendritic spines and measure their relationship to both existing and other newly formed spines, inferring important information about how SRGAP2 affects the clustering of excitatory synapses. We find that sensory deprivation *via* bilateral whisker trimming causes new spines to preferentially form away from pre-existing spine clusters in SRGAP2 knockdown mice, but not in WT. Additionally, we find that SRGAP2 knockdown induces spines to form in larger clusters than in WT animals.

## Introduction

The clustered plasticity model states that neighboring spines tend to transmit similar information to the postsynaptic neuron (Fu et al. 2012, Govindarajan et al. 2006, Larkum et al. 2008). For example, adjacent spines are frequently synchronized in spontaneous active networks in both hippocampal CA3 and in the barrel cortex (Takahashi et al. 2012). Additionally, synapses of intralaminar inputs cluster onto the basal dendrites of layer 5 pyramidal neurons, with 4 to 14 synapses often occurring within a <30  $\mu\text{m}$  segment of dendrite (Gökçe et al. 2016). As an example in a sensory area, in mouse auditory cortex, while spines respond to different

frequencies are frequently interspersed, 26% of neighboring spines are tuned for similar effective frequencies, much more frequently than expected from random chance (**Chen et al. 2011**). Such clustering of synaptic inputs and plasticity may be a way for neural networks to compartmentalize correlated inputs along dendrites, reifying the ability of neurons to nonlinearly integrate correlated synaptic inputs (**Larkum et al. 2008, Stuart and Spruston 2015**). There is also evidence of non-uniform distributions of spine density across a given dendrite, suggesting that the local clustering of dendritic spines is important to dendritic integration and function (**Druckmann et al. 2014, Larkum et al. 2008, Stuart and Spruston 2015, Yadav et al. 2012**).

The clustering of synapses can also allow these correlated inputs to promote potentiation of each other. In hippocampus, LTP at individual synapses reduces the threshold for potentiation at neighboring synapses within a  $\sim 10\ \mu\text{m}$  segment of dendrite, an effect that lasts  $\sim 10$  minutes (**Harvey and Svoboda 2007**). A possible behavioral correlate of this effect can be seen in motor cortex when mice are trained in a seed-reaching task. On apical dendrites of layer 5 pyramidal cells under these conditions,  $\sim 1/3$  of new spines that formed during the acquisition phase of task learning (days 1-4) emerged in clusters, defined either as two or more neighboring spines formed with no interspersed existing spines or (more liberally) as two or more spines that form within  $5\ \mu\text{m}$  of each other (**Fu et al. 2012**). In contrast, in untrained control mice or trained mice during the consolidation phase of learning (days 13-16), fewer new spines form in clusters (**Fu et al. 2012**).

As SRGAP2 is involved in controlling the distribution of inhibitory synapses between dendritic shafts and dendritic spines, and regulates both inhibitory and excitatory synapses, it may also be involved in regulating the clustered formation of dendritic spines. However, no studies have yet examined this role.

To investigate how SRGAP2 affects the clustering of plastic excitatory synapses in adults, we imaged spines on the apical dendrites of layer 5 pyramidal neurons in the barrel cortex of adult mice ( $P > 150$ ). We observed existing and newly-formed spines in both WT and SRGAP2 knockdown animals before and after whisker trimming or sham treatment. As our results did not detect any difference between baseline measurements and those obtained after sham treatment, we pooled the data for increased statistical power, as newly formed spines represent a small proportion of the spines we imaged. Therefore, our spine measurements are reported in four conditions - WT animals before and after whisker trimming, and SRGAP2 knockdown animals before and after whisker trimming (WT baseline, WT trim, MUT baseline, and MUT trim).

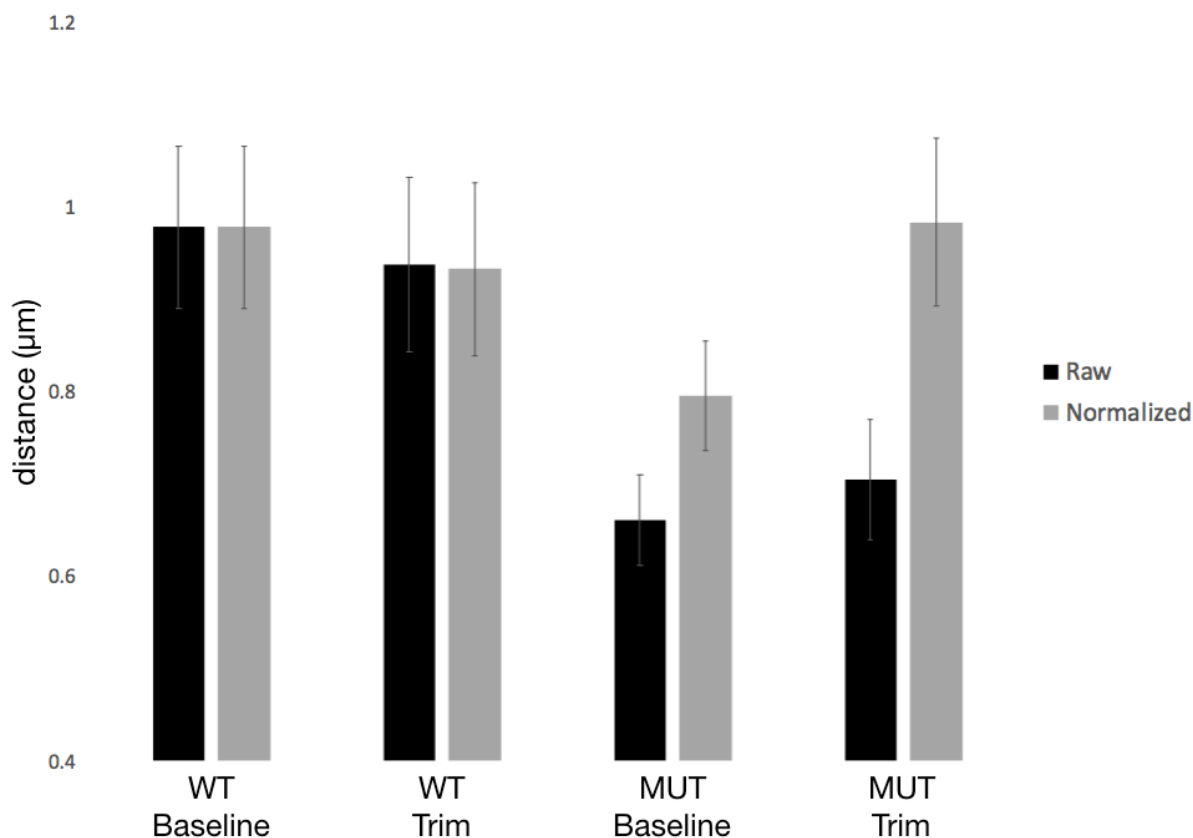
## Results

### *SRGAP2 knockdown does not affect the local density of where new spines form at baseline*

To determine whether SRGAP2 knockdown changes where new spines preferentially form on a dendrite, we first measured the distance between a newly formed spine and the closest pre-existing spine. As SRGAP2 knockdown increases the spine density in layer 5 apical dendrites (see Chapter 3), this will increase the likelihood of new spines forming near existing spines. Therefore, to account for the differential in density and ensure that changes in the distances we measure are not simply due to changes in density, we normalized our measurement by dividing each count by the ratio between each condition's density and that of the WT baseline condition. We find that SRGAP2 knockdown does not affect distance from newly formed spines to their nearest neighbor (**Figure 4.1** shows both raw and normalized data; normalized distances - WT =  $0.98 \pm 0.09 \mu\text{m}$ , MUT =  $0.80 \pm 0.06 \mu\text{m}$ ;  $p=0.814$ , Kruskal-Wallis test).



## Distance to Nearest Neighbor for Newly Formed Spines

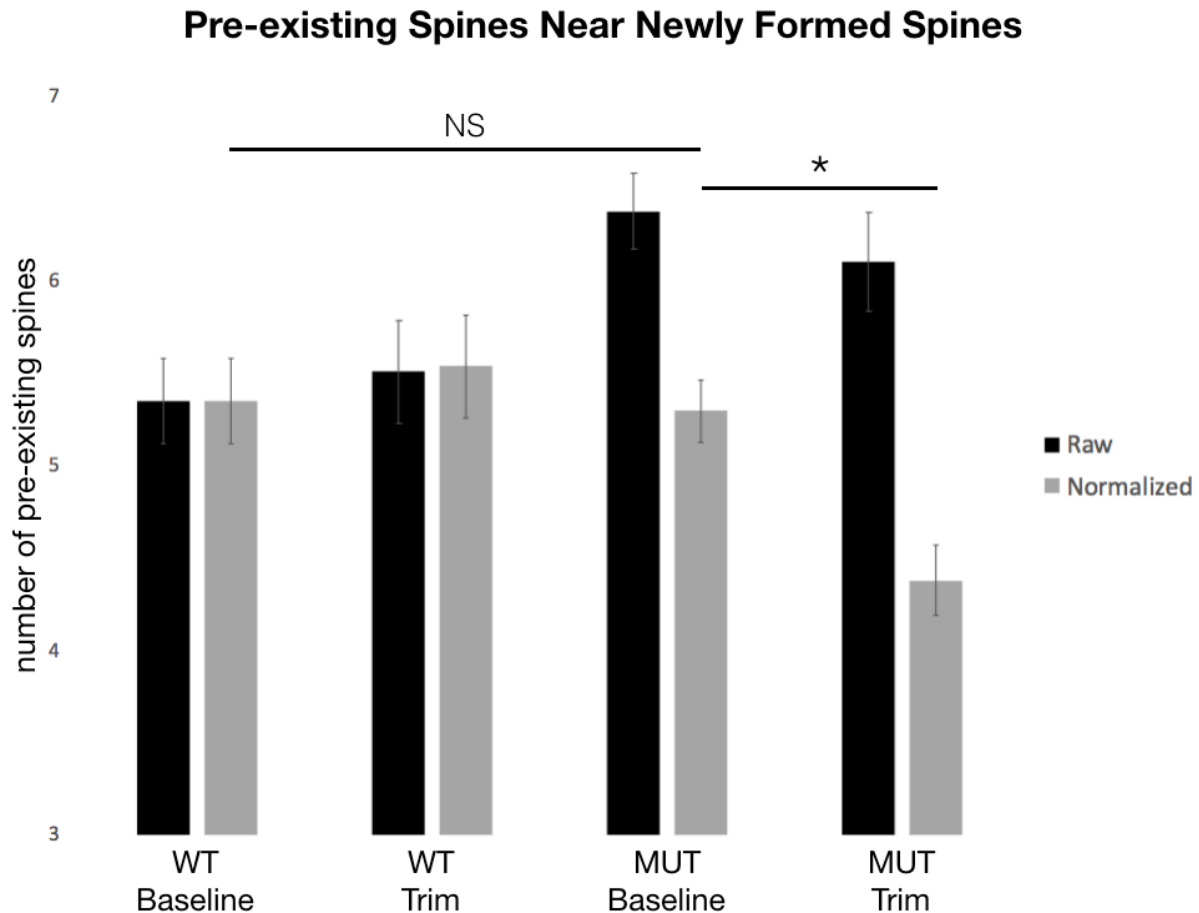


**Figure 4.1 - Distance from newly formed spines to closest pre-existing spines in layer 5 apical dendrites in adult WT and SRGAP2 KD mice**

Distance is normalized for each condition by dividing by the ratio of the density of the condition to the density of the WT baseline. Data represents mean  $\pm$  SEM.  $p=0.814$ , Kruskal-Wallis test (comparison between normalized data). Sample sizes (spines):  $n_{WTbaseline} = 246$ ,  $n_{WTtrim} = 203$ ,  $n_{MUTbaseline} = 197$ ,  $n_{MUTtrim} = 226$ .

Next, we measured the local density of existing spines where new spines form by counting the existing spines within  $5\mu m$  of a newly formed spine. As with the distance to nearest neighbor, we accounted for density differences by normalizing each count by the ratio between a condition's density and that of the WT baseline condition. We find that SRGAP2 knockdown does not affect the local density of dendrites where new spines form (**Figure 4.2**; normalized

data - WT =  $5.35 \pm 0.23$ , MUT =  $5.29 \pm 0.17$  existing spines within  $5 \mu\text{m}$ ;  $p=0.446$ , Kruskal-Wallis test followed by Mann-Whitney U test).



**Figure 4.2 - Number of pre-existing spines within  $5 \mu\text{m}$  of newly formed spines in layer 5 apical dendrites in adult WT and SRGAP2 KD mice**

Number of spines is normalized for each condition by dividing by the ratio of the density of the condition to the density of the WT baseline. Data represents mean  $\pm$  SEM. \* $p<0.001$ , NS  $p=0.446$ , Kruskal-Wallis test followed by Mann-Whitney U test. Comparisons performed between normalized data. Sample sizes (spines):  $n_{\text{WTbaseline}} = 246$ ,  $n_{\text{WTtrim}} = 203$ ,  $n_{\text{MUTbaseline}} = 197$ ,  $n_{\text{MUTtrim}} = 226$ .

***After whisker trimming, SRGAP2 knockdown mice form spines in sparser dendritic regions***

To determine whether whisker trimming affects where new spines preferentially form on a dendrite, we compared the distance between newly formed spines and their nearest pre-existing

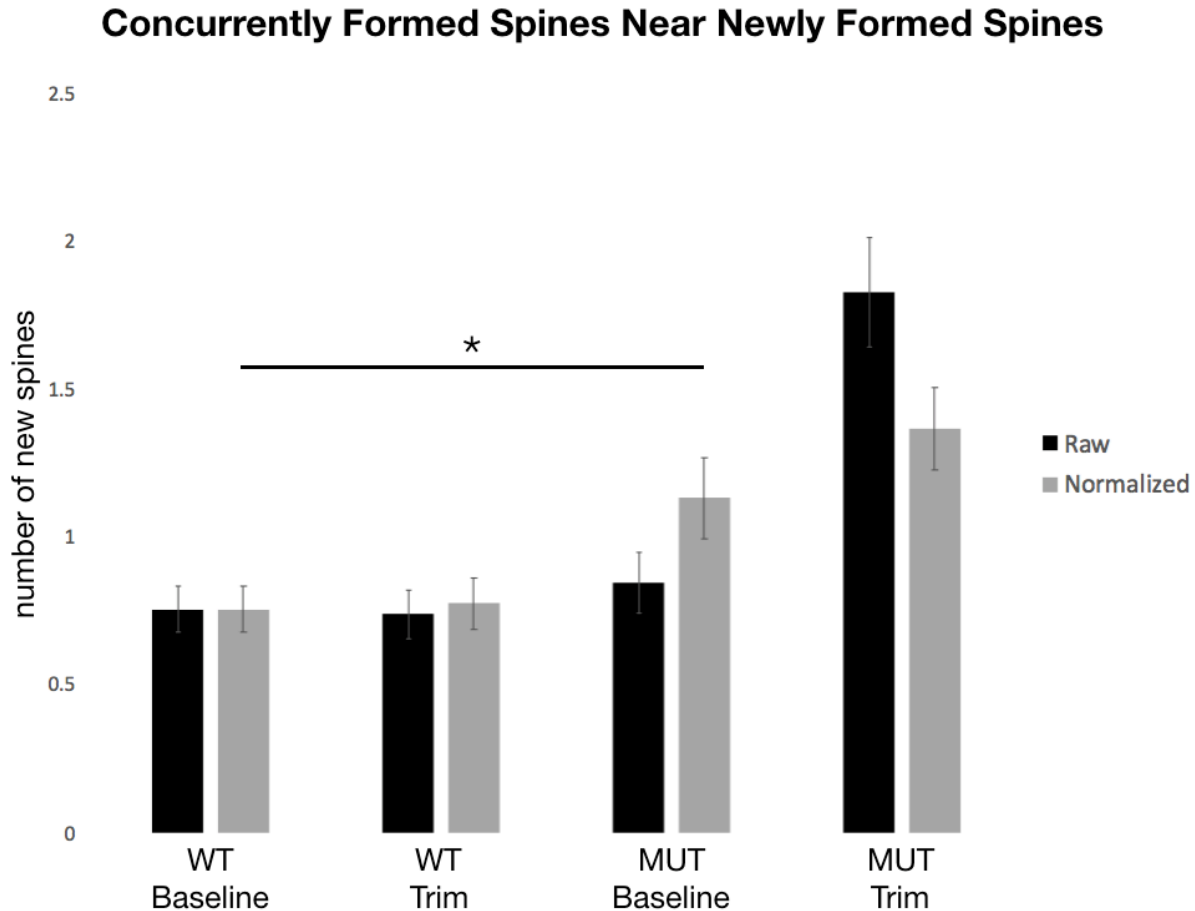
neighbor before and after bilateral whisker trimming in both WT and SRGAP2 knockdown conditions. We find that whisker trimming does not affect the distance from newly formed spines to their nearest neighbor (**Figure 4.1**; normalized distances - WT Baseline =  $0.98 \pm 0.09 \mu\text{m}$ , WT Trim =  $0.93 \pm 0.09 \mu\text{m}$ , MUT Baseline =  $0.80 \pm 0.06 \mu\text{m}$ , MUT Trim =  $0.98 \pm 0.09 \mu\text{m}$ ;  $p=0.814$ , Kruskal-Wallis test).

As above, we then compared the local density of existing spines where new spines form before and after whisker trimming for WT and SRGAP2 knockdown mice. In WT animals, we see no difference in the local density of dendrites where new spines form before and after bilateral whisker-trimming (**Figure 4.2**; normalized data - WT Baseline =  $5.35 \pm 0.23$ , WT Trim =  $5.54 \pm 0.28$  existing spines within  $5 \mu\text{m}$ ;  $p=0.417$ , Kruskal-Wallis test followed by Mann-Whitney U test). However, in SRGAP2 knockdown animals, we note a significant decrease in the number of existing spines within  $5 \mu\text{m}$  of a given newly formed spine (**Figure 4.2**; normalized data - MUT Baseline =  $5.29 \pm 0.17$ , MUT Trim =  $4.38 \pm 0.19$  existing spines within  $5 \mu\text{m}$ ;  $p<0.001$ , Kruskal-Wallis test followed by Mann-Whitney U test).

### ***SRGAP2 knockdown induces larger clusters of newly formed spines***

Next, to determine whether new spines form in clusters in adult WT or SRGAP2 knockdown mice, we measured the number of other new spines that form within  $5 \mu\text{m}$  of a newly formed spine. To account for the increased probability that newly formed spines will form closer to each other simply due to increased formation rates, we normalized our counts for each condition by dividing each count by the ratio between its formation rate (see Chapter 3) and that of the WT baseline condition. Compared to WT, we find that SRGAP2 knockdown animals form spines in

larger clusters (**Figure 4.3**; normalized data - WT Baseline =  $0.76 \pm 0.08$ , MUT Trim =  $1.14 \pm 0.14$  new spines within  $5 \mu\text{m}$ ;  $p < 0.002$ , Kruskal-Wallis test followed by Mann-Whitney U test).



**Figure 4.3 - Number of concurrently formed spines within  $5 \mu\text{m}$  of newly formed spines in layer 5 apical dendrites in adult WT and SRGAP2 KD mice**

Number of spines is normalized for each condition by dividing by the ratio of the formation rate of the condition to the formation rate of the WT baseline. Data represents mean  $\pm$  SEM. \* $p < 0.02$ , Kruskal-Wallis test followed by Mann-Whitney U test. Comparisons performed between normalized data. Sample sizes (spines):  $n_{\text{WTbaseline}} = 246$ ,  $n_{\text{WTtrim}} = 203$ ,  $n_{\text{MUTbaseline}} = 197$ ,  $n_{\text{MUTtrim}} = 226$ .

We then wanted to determine whether sensory deprivation affects the clustered formation of dendritic spines differently in WT and SRGAP2 knockdown animals. We find that whisker trimming in WT animals does not affect the clustered formation of new spines (**Figure 4.3**;

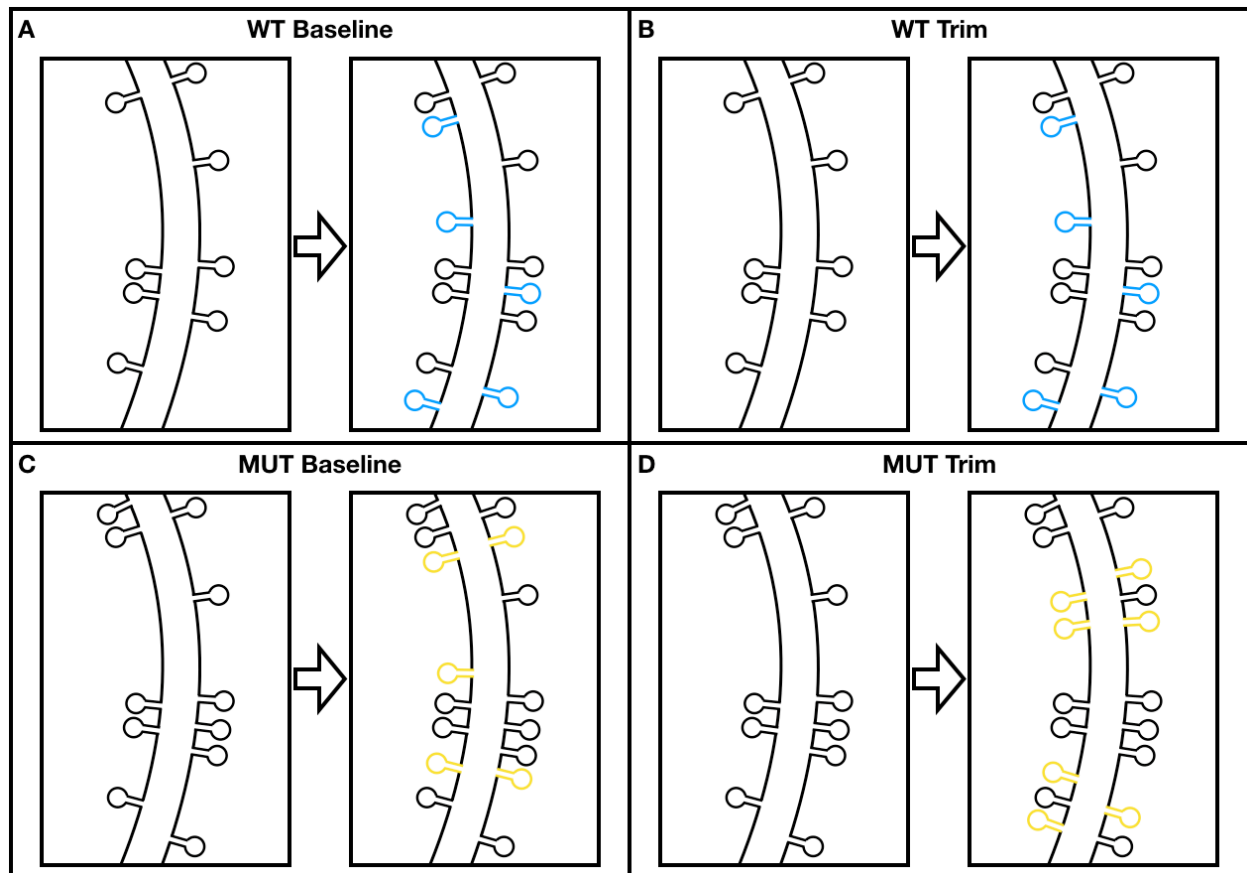
normalized data - WT Baseline =  $0.76 \pm 0.08$ , WT Trim =  $0.78 \pm 0.09$  new spines within 5  $\mu\text{m}$ ;  $p=0.08$ , Kruskal-Wallis test followed by Mann-Whitney U test). Whisker trimming in SRGAP2 knockdown animals may induce the formation of larger clusters than at baseline, but the increase we observe does not reach significance (**Figure 4.3**; normalized data - MUT Baseline =  $1.14 \pm 0.14$ , MUT Trim =  $1.37 \pm 0.14$  new spines within 5  $\mu\text{m}$ ;  $p=0.058$ , Kruskal-Wallis test followed by Mann-Whitney U test).

## Discussion

Here, we measure the clustering of newly formed spines in adult ( $P>150$ ) mice in both WT and SRGAP2 knockdown conditions in the apical dendrites of layer 5 pyramidal neurons. We find that knocking down SRGAP2 induces the formation of larger spine clusters than WT mice. In addition, we find that SRGAP2 knockdown causes new spine clusters to form preferentially in dendritic locations that have less local density in response to sensory deprivation via bilateral whisker trimming (**Figure 4.4**).

Interestingly, while new spines form preferentially in sparser regions after whisker trimming in SRGAP2 knockout animals, the distance of new spines to the nearest existing neighbor does not change (**Figure 4.1**). This suggests that newly formed spines still preferentially form in locations where there are already synapses, rather than “empty” areas along dendrites. As clustered spines tend to share similar inputs (**Fu et al. 2012, Govindarajan et al. 2006, Larkum et al. 2008**), it is possible that the newly formed clusters share correlated presynaptic activity as the existing spine(s) they form near. Therefore, it is possible that existing spines that do not already exist in functional clusters may serve as “anchors” or attractors for new clusters of correlated spines. This could provide a mechanism for synaptic tagging and

capture, whereby increased activation of the existing synapses could stimulate a local environment of increased translation, causing a cascade of events leading to the formation of new spines, possibly at the site of “silent” synapses (Govindarajan et al. 2006, Kerchner and Nicoll 2008).



**Figure 4.4 - SRGAP2 knockdown’s effect on clustered spine formation in in layer 5 apical dendrites of adult barrel cortex**

Schematics of spine formation in **A. WT Baseline**, **B. WT Trim**, **C. MUT Baseline**, and **D. MUT Trim** conditions.

SRGAP2 knockdown induces formation of spine clusters with more spines than in WT animals. In addition, whisker trimming induces spine cluster formation away from existing spine clusters in SRGAP2 knockdown animals, but not in WT mice. Spine elimination is not represented in these schematics.

To test this possibility, functional imaging experiments can be performed to monitor activity in these spines, e.g. through calcium imaging in dendritic spines (Chen et al. 2013).

Such experiments can track the formation of new spines while simultaneously imaging existing spines nearby while measuring their activity, allowing us to determine whether their input is correlated.

Similarly, while other studies show that formation of dendritic spines in motor cortex following motor learning can be limited to specific dendritic branches (**Yang et al. 2014**), it is unclear how the local milieu of existing spines affects new spine formation. Future experiments should examine the relationship between existing spines and newly formed clusters, as we performed here for barrel cortex.

While we performed our experiments in adult ( $P > 150$ ) mice, it is unclear to what degree clustered spine formation occurs in younger animals. Similarly, it is unknown whether our results can be generalized across other sensory cortical areas. Future work should determine whether the effects of SRGAP2 knockdown that we observe here are seen in other conditions.

Finally, while we examined how SRGAP2 affects the clustering of spine formation, it remains unclear whether spine elimination is also clustered. Future work should determine whether synapse pruning preferentially eliminates clusters of synapses, and what the functional consequences are. For example, it is possible that a new cluster of spines forms next to an existing “anchor” synapse, as described above. It is possible that some or all of these spines will fail to acquire PSD-95 and become functional synapses, which strongly increases the likelihood of rapid elimination (**Cane et al. 2014**). It will be important to determine whether these newly formed spines are likely to be eliminated altogether, and whether the pre-existing synapse is spared or is eliminated with its compatriots as a sort of failed test balloon.

## **CHAPTER 5**

### Conclusions



In this dissertation, we examined the consequences of knocking down SRGAP2 on spine dynamics in adult mouse neocortex using longitudinal *in vivo* structural imaging. Previous studies showed that SRGAP2 knockdown delays maturation in young adult mice (P~60-75) by promoting neoteny and extending the amount of time for spine head size to reach their growth potential, while also increasing spine neck length and increasing spine density overall (**Charrier et al. 2012, Fossati et al. 2016**). Here, we show that the increase in spine density in layer 5 pyramidal cells of the barrel cortex extends at least through the first five months of the mouse lifespan. Additionally, we show that SRGAP2 decreases the relative turnover rate of spines, and increases their potential to respond to environmental changes (bilateral whisker trimming). Finally, we examined the effect of SRGAP2 knockdown on spine clustering, and find that SRGAP2 knockdown increases clustered formation of new spines and induces the preferential formation of new spine clusters in sparse dendritic locations.

### **SRGAP2 and spine dynamics in other brain regions**

The dynamics of the dendritic segments we determined thus far are restricted to the apical dendrites of layer 5 pyramidal neurons in the barrel cortex. However, there is heterogeneity at all levels in cortical pyramidal neurons, and it is important to determine differences at these levels. While it is possible that our results are generalizable throughout the cortex, it is likely that different regions are affected differently by SRGAP2. Indeed, SRGAP2's evolution likely affected specific brain areas, and determining how and where its evolution effected changes is critical to divining the deeper mysteries of human brain evolution.

It will also be important to determine SRGAP2 manipulation's effect on non-neocortical regions, such as hippocampus, where spine dynamics are different (**Attardo et al. 2015**). Future

work should examine SRGAP2 in the context of several brain areas (e.g., sensory cortex, motor cortex, associational cortices, hippocampus) and in different cellular/subcellular populations within those areas. These properties can be determined both in fixed time-point experiments, as done previously (**Charrier et al. 2012, Fossati et al. 2016, Guerrier et al. 2009**), and in longitudinal *in vivo* imaging experiments to determine synaptic dynamics, as shown in this dissertation.

Additionally, as pyramidal neurons of different cortical layers have a plethora of morphological and functional differences (**Harris and Shepherd 2015**), it is likely that cells of different layers will be differentially regulated by SRGAP2, as they display different spine properties and dynamics at baseline (**Tjia et al. 2017**). Neurons are also made up of several delineable subcellular compartments, each of which can display different spine properties (**Sala and Segal 2014**). It will be necessary for future experiments to determine how SRGAP2 regulates synapses in each of these dendritic populations.

### **SRGAP2 and inhibitory synaptic dynamics**

As SRGAP2 is now known to regulate inhibitory synapses along with excitatory synapses (**Fossati et al. 2016**), its effect on inhibitory synapse dynamics need to be determined. The use of a reporter-marked gephyrin has now been used in several studies to longitudinally monitor inhibitory synapses (e.g. **Chen et al. 2012, Villa et al. 2016**), and is a technique that can be used to study inhibitory synapses in the context of SRGAP2 manipulation.

As SRGAP2 coregulates inhibitory and excitatory synapses, experiments tracking both types of synapses simultaneously (e.g., using both marked gephyrin and PSD-95) will be particularly illuminating. Targeted manipulation of specific domains on SRGAP2 (e.g.,

disrupting the F-BAR domain as in **Fossati et al. 2016**) will allow us to target specific functions. For example, disrupting the ability of SRGAP2 to bind Homer should lead to neoteny in excitatory synapses; whether inhibitory synapse dynamics are affected in this case could reveal the impact of homeostatic plasticity versus cell-autonomous effects on SRGAP2 regulation on synaptic dynamics and the excitatory-inhibitory balance.

Our results show that SRGAP2 knockdown decreases the turnover rate of dendritic spines in adult mice. However, it is unclear how inhibitory synapse dynamics are affected. Inhibitory synapse dynamics differ from those of dendritic spines, and are also different between inhibitory shaft synapses and inhibitory spine synapses (**Chen et al. 2012**). SRGAP2 knockdown is known to increase the proportion of inhibitory synapses in spines compared to shafts, allowing for more effective compartmentalization of inhibition (**Chiu et al. 2013, Fossati et al. 2016**). In layer 2/3 neurons of the visual cortex, inhibitory spine synapses are much more dynamic than inhibitory shaft synapses (**Chen et al. 2012**). In addition, inhibitory spine synapses were exclusively located on stable, persistent spines (**Chen et al. 2012**). It is possible that the decreased spine turnover we observe with SRGAP2 knockdown is correlated with an accompanying decrease in the turnover of inhibitory spine synapses, as increased stability in inhibitory spine synapses may in turn increase stability of their resident spines. It will be important to determine whether this decrease in spine turnover is seen if SRGAP2 is replaced with a mutant form which is unable to affect inhibitory synapses (**Fossati et al. 2016**).

Additionally, we note that SRGAP2 knockdown induces the formation of larger spine clusters and can change the dendritic location where new clusters form in response to environmental changes. Recently, Bloss et al. showed that inhibitory interneurons can target very specific dendritic compartments (**Bloss et al. 2016**). Additionally, in layer 2/3 pyramidal neurons

of the visual cortex, inhibitory synapses and dendritic spines exhibit clustered plasticity together (**Chen et al. 2012**). Future work could elucidate the distribution of inhibitory synapses relative to these newly formed clusters of synapses, as well as the dynamics of inhibitory synapses as well. For example, as GABAergic stimulation of dendritic shafts can promote the elimination of local dendritic spines (**Hayama et al. 2013**), and SRGAP2 knockdown reduces the proportion of inhibitory shaft synapses (**Fossati et al. 2016**), it is possible that whisker trimming in SRGAP2 knockdown animals promotes the formation of spine clusters in locations with few previous spines by simultaneously reducing the number of inhibitory synapses in these areas to prime the segment, allowing the strengthening of new connections.

### **SRGAP2C in adults**

While studies on SRGAP2C have thus far shown that it inhibits every known function of SRGAP2A, it is possible that SRGAP2A has heretofore unknown effects that cannot be blocked by SRGAP2C. Future work will need to address whether SRGAP2C overexpression has the same effects that we see when knocking down SRGAP2 in adult mice.

SRGAP2C may also be expressed differently at later ages in humans than in adolescence. As it promotes neoteny, it is possible that SRGAP2C expression levels lower throughout development, and hits low levels in adulthood. To properly model human-like brain development, it will be important to selectively knock down SRGAP2 or overexpress SRGAP2C in a manner that matches the temporal expression of SRGAP2C in humans (see below on temporally and spatially specific manipulation of SRGAP2).

## **Behavioral and cognitive consequences of SRGAP2 manipulation**

While we hypothesize that SRGAP2 knockdown or SRGAP2C overexpression imbues synapses with a more human-like phenotype, it remains unclear how these effects translate to behavioral and cognitive consequences. There are two mutually non-exclusive possibilities immediately apparent - the delayed maturation may allow affected older animals to learn tasks as efficiently as their younger compatriots, and affected young animals may perform better on behavioral tasks compared to wild-type animals. Both of these possibilities should be tested in future work.

In our experiments, we observe a significant increase in spine formation in response to whisker trimming in SRGAP2 knockdown animals compared to wild-type. This suggests that the network may be more responsive to environmental changes when SRGAP2 is knocked down. As SRGAP2 is expressed throughout neocortex and other areas, we might expect a wide variety of behavioral consequences if our results hold in other regions. In the motor cortex, increased remodeling in response to training might promote faster learning in motor tasks (e.g. **Tjia et al. 2017**), while increased spine formation in the hippocampus might lead to improved performance in spatial (e.g. the Morris Water Maze) and contextual (e.g. contextual fear conditioning) memory tasks.

When observing the dynamics of dendritic spines, it is unclear whether the dynamics are the cause of consequence of behavior changes (**Gipson and Olive 2017**). Therefore, it will be necessary to specifically affect dendritic spines and determine the effect that has on behavioral readouts. For example, selectively shrinking motor cortex spines that are enlarged by motor skill training can disrupt the ability of the animal to learn the task (**Hayashi-Takagi et al. 2015**).

Additionally, functional imaging of dendritic spines and inhibitory synapses can now resolve subcellular responses, help elucidate their functions in various assays. For example,

separating the dendritic arbor of a given neuron in primary visual cortex into multiple domains and analyzing them individually shows differential responses to multiple tested visual orientations; on a population level, these responses predict the orientation selectivity of the cell (**Chen et al. 2013**). Similar experiments can elucidate circuit wiring, inputs from various stimuli, plasticity, and integration of multiple sources.

In the context of our results, functional imaging of dendritic spines during whisker trimming may reveal the logic behind circuit reorganization. For example, it is possible that whisker trimming induces decreases inhibitory activity in dendritic segments without many existing spines in SRGAP2 knockdown mice. Functional imaging allow us to determine the excitatory and inhibitory activity that takes place in these dendrites during spine formation. Additionally, training mice in a whisker-related task and functionally imaging the barrel cortex could reveal selective stabilization of newly formed spines which are active during learning, and may be correlated with the ability of the animal to successfully learn the task (see **Kuhlman et al. 2014**). If SRGAP2 knockdown increases formation in response to learning, it will be interesting to whether this promotes more task-related spines to be generated and stabilized, leading to an increased ability to learn.

### **Temporally and spatially specific manipulation of SRGAP2**

While we examined the effects of lifelong SRGAP2 knockdown in adults, it is unclear whether what we observe is due to developmental compensation for SRGAP2 loss, or due directly to loss of SRGAP2 function. To distinguish between these possibilities, future experiments should conditionally knockout SRGAP2 in both temporally and spatially restricted manners.

To determine whether loss of SRGAP2 function in adults can have similar effects to what we observe (i.e., increased spine density and reduced spine turnover), SRGAP2 could be knocked down in adults through an inducible reporter. For example, a SRGAP2 conditional knockout line (with the SRGAP2 gene flanked by loxP sites which can be removed by Cre recombinase) can have SRGAP2 knocked down in adulthood by using an inducible Cre line (see e.g. **Ramirez et al. 2013**). Longitudinal imaging can then elucidate what occurs following SRGAP2 knockdown. In addition, to determine whether these effects are cell-autonomous, SRGAP2 should be knocked down sparsely (e.g., utilizing sparse expression through promoters like Thy1, or sparse viral delivery methods) to confine its effects to the observed neuron.

### **Improvements in dendritic spine imaging**

Currently, *in vivo* imaging of dendritic spines is very labor intensive, limiting the amount of data that can reasonably be collected and analyzed. However, much work is currently being done on the automation of spine detection from 2PSLM stacks (**Polleux lab, unpublished results, Singh et al. 2017**).

Additionally, new microscopy techniques can improve the characterization of dendritic spines *in vivo*, as well as allow the imaging of multiple areas simultaneously (**Szalay et al. 2016**). For example, super-resolution imaging may soon allow for measurements of spine head and neck dimensions *in vivo*, allowing us to examine changes in individual spines over time (**MacGillavry and Hoogenraad 2015, Maiti et al. 2015, Tønnesen et al. 2014**). Importantly, this will allow us to determine the effect of SRGAP2 manipulation on individual spines by observing spines before and after SRGAP2 knockout (see above on temporally and spatially specific manipulation of SRGAP2). Additionally, we could compare the effects of SRGAP2

manipulation in multiple brain regions simultaneously (e.g., motor and barrel cortex, in response to a whisker-related motor task; **Kuhlman et al. 2014, Szalay et al. 2016**).

In summary, in this thesis we performed the first analysis of SRGAP2 function in adult mice and its role in synaptic dynamics. Our results suggest that SRGAP2 knockdown can model human-like neoteny past previously reported ages (from P<75 in previous studies to P>150 here) and that SRGAP2 regulates the clustered formation of synapses. Taken together, these results represent a key stepping stone in determining how SRGAP2 regulates neural circuitry and plasticity, and may lead to further understanding of human brain evolution.



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